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HIGH INCIDENCE OF INTRAPERITONEAL RHABDOMYOSARCOMAS IN RATS AFTER GROWTH AND REGRESSION OF *NOSEMA CUNICULI* INFECTED YOSHIDA ASCITES SARCOMA

By

MICHAEL PETRI

Received 14 viii 67

Previous works (*Petri* 1965 1966) have presented some observations on the Yoshida rat ascites sarcoma infected by the intracellular microsporidian parasite *Nosema cuniculi* previously known as *Encephalitozoon cuniculi* In this laboratory such a tumour has been carried by serial intraperitoneal passages continuously for several years during which malignancy capacity for cellular proliferation and ability to metastasize were maintained It was mentioned that malignancy however is somewhat diminished especially evident by an increased survival rate as compared to the uninfected sarcoma

The purpose of the present paper is to record the findings in rats surviving inoculation of the infected sarcoma when left untreated until they die a high incidence of intra abdominal rhabdomyosarcomas has been found at autopsy The nosema infected ascitic sarcoma develops but then disappears leaving a fibrous peritoneal cavity There is a latent period until the late tumour is found

MATERIAL AND METHODS

Rat strain tumour and transplantation see *Petri* (1966)

The rats are kept singly in wired cages They are offered daily fresh supplies of water and the following diet (standard breeding diet of the National Vitamin Laboratory)

Skim milk powder	34 4 per cent
Coarsely ground rye flour	34 3
Wheat bran	11 4 „
Dry yeast	8 6 „
Peanut oil	11 4

To which is added 40 I U of vitamin A per gram corresponding to about 4 5 I U of vitamin A per gram of the diet and vitamin D 150 I U per 17 5 kg corresponding to 0 01 I U *per gram of the diet*

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A small number of the rats employed have been rachitic for a period of about 1 month after birth and used for biological assay of vitamin D The actual number of these rats has been recorded but is estimated to be about 25 per cent of the total number

Histological Procedure

Fixation of thin slices by phosphate buffered 4 per cent formaldehyde or by Lillie s acetic alcohol formaldehyde mixture at 4 C for approximately 48 hours

Staining by haematoxylin erythrosin van Gieson haematoxylin Mallory s trichome method Giemsa s stain for sections after *Wohlbach* (see *Lillie* 1966) Gram stain for sections using Hucker Conn crystal violet with acetone differentiation slightly modified from *Lillie* (1966)

RESULTS

Incidence

In the course of 4 years and 7 months 278 passages were made by inoculating 2 rats every 5 to 7 days with 0 5 to 0 7 ml parasitized sarcoma a total of about 10⁸ cells of which about 10 per cent were infected by Nosema altogether 556 rats

According to our earlier report (*Petri* 1966) on a smaller group of rats (131 animals) the survival rate was found to range at 18 per cent Among the 556 animals now recorded 80 survived (more than 5 months) which is 14 per cent Out of these 12 were still alive when the present manuscript was written Forty three of the survivors were kept untreated in their cages until they died and were autopsied 16 sarcomas were found excluding two lymphosarcomas an incidence of 37 per cent

The mean survival time i e the time from inoculation of the infected sarcoma till death of the rat in which the new tumour had developed was 18 1 months ranging from 12 to 22 months in the 13 cases while in two cases the time was 5 and 8 months In the group of rats which died without tumours the survival time was 17 9 months Although the ages of the animals used were not recorded this finding seems to indicate that the late tumours found did not shorten the lives of the tumour bearing animals

Description of Tumours

A few benign tumours of well recognized occurrence in rats (see *Snell* 1965) were found one chromofobe pituitary adenoma one adrenal adenoma and one mammary fibroadenoma None occurred in rats with malignancies

The two cases classified as lymphosarcomas were located to the mesentery or mediastinum

The rest of the tumours were by naked eye inspection found between the agglutinated intestinal loops either as one large single tumour or with satellites in the vicinity The general impression was that they had [illegible] the fibrous tissue which glues the serosal surfaces

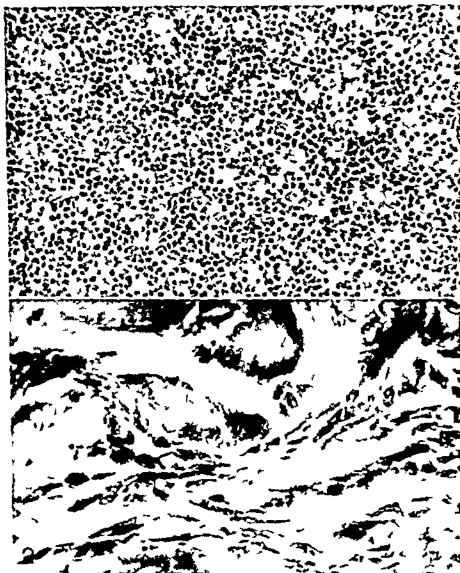


Fig 1 Starry sky lymphoma Haematoxylin erythrosin 2[illegible]0 ×
Fig 2 Cross striation Giemsa 880×

together as late sequelae of the infected sarcoma *i e* after its regression (see *Petri* 1966) The macroscopic appearance suggested a malignant tumour considerable necroses of the central parts with haemorrhage and caseation while the cut surface of viable parts of the tumour was either fleshy grey moist and rather friable or whitish fibrous partly whorled They were all non encapsulated

Although the tumours in two cases had invaded the muscular layer

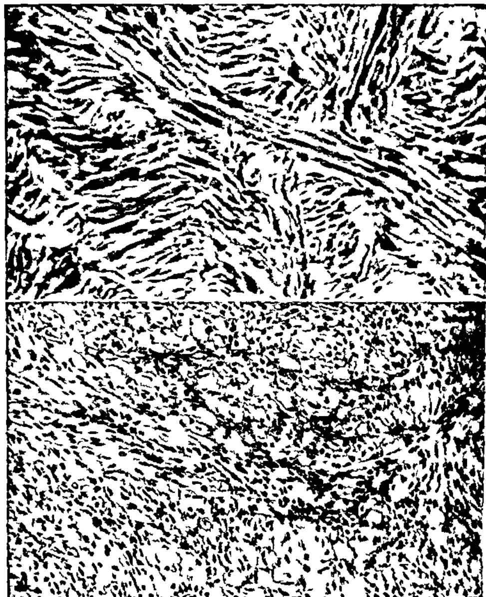


Fig 3 Muscle fibers Haematoxylin erythrosin 220 ×
Fig 4 Muscle fibers Van Gieson 220 ×

of the anterior abdominal wall invasion of the posterior muscular wall diaphragm or large paraspinal muscles was not observed Thus in the majority of cases evidence of connection with cross striated muscle tissue was not found

Metastases to mediastinal lymph nodes were noted in the two cases in which they were searched for

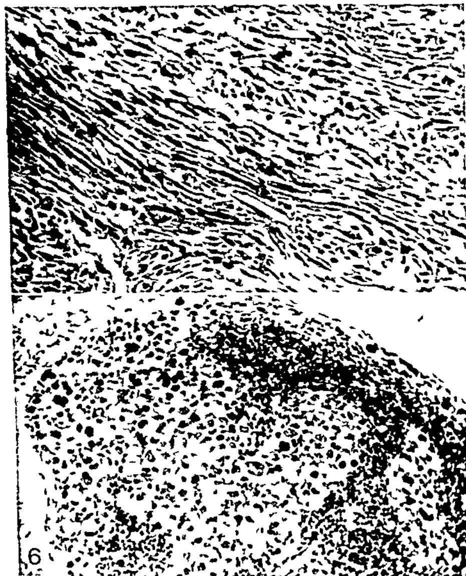


Fig 5 Primiti e mesenchymal area Giemsa 2 0 ×
Fig 6 Pleomorphic transplanted sarcoma in a ion of mesenteric lymph node Haematoxylin erythrosin 220 ×

HISTOLOGY

A *Lymphosarcomas* The 2 lymphosarcomas found were of the so called starry sky pattern (see *Snell* 1965) closely aggregated lymphocytes with single large reticular cells scattered as stars in the sky (Fig 1) The cytoplasms of these cells contained nuclear debris By Gram staining no *Nosema cuniculi* were found

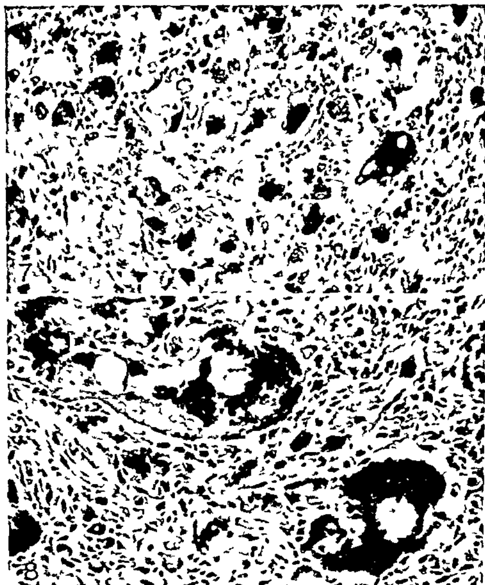


Fig 7 Pleomorphic cells with foamy cytoplasm Van Gieson 220 ×
Fig 8 Giant cells Van Gieson 220 ×

B *Spindle cell sarcomas and myosarcomas* 16 cases were found among which 11 were myosarcomas of various degrees of anaplasia 6 of these displayed areas with undubious occurrence of typical skeletal muscle cells with longitudinal and occasional cross striations (Figs 2–4) Some parts were made up of fibrous tissue with fibroblasts and collagenic fibres resembling hard fibrous scar like tissue or a highly differentiated fibrosarcoma Tissue of primitive mesenchymal charac ter was also observed in which stellate cells were dispersed in a homo

genous matrix (Fig 5) Like the amorphous ground substance of undifferentiated mesenchyme it contained acid mucopolysaccharides revealed by its staining with alcian blue

In other areas the histological picture was one of polymorphism and anaplasia (Figs 6–7) A particular feature was multinucleated cells of enormous size (Fig 8)

Transplantation

One tumour was transplanted by mincing non necrotic parts with scissors and by injection of the minced matter into the peritoneal cavity using a wide bore needle In several cases penicillin and streptomycin or sulfonamides were given during the first days by intraperitoneal injection The tumour was a highly pleomorphic sarcoma representative areas of which are shown in Figs 6–7 It was present in all passages within the peritoneal cavity in 2 cases as a large non encapsulated growth in 5 cases as multiple grape like nodules with widespread necrosis In all passages in which tumour tissue developed 2 to 5 ml of blood stained ascites were found at autopsy but in Giemsa stained smears Yoshida sarcoma cells were not observed Likewise intraperitoneal injection of 1 ml of ascites into 6 rats was not followed by development of ascitic or solid tumours

Control Animals

A *Untreated rats* The incidence of spontaneous tumours in the strain of rats used is not known and the rats are not strictly inbred though mating between brother and sisters is generally attempted It dates back to 1919 (see *Petri* 1966) When the incidence of tumours of presumably one type was realized to be high a series of control animals of this strain was introduced These rats were kept untreated under the same conditions and given the same food At the time of submission of the present manuscript for publication autopsies had been performed on 20 aged from 2 years to 4 years and 1 month with an average of 2 years and 6 months in which no tumours were found

B *Nosema infected* rats Ascites with nosema infected cells but without Yoshida sarcoma cells was injected intraperitoneally into 25 rats utilizing the ascites formed in mice after injection into the peritoneal cavity of nosema containing material This infected ascites can be carried in serial passages In the mouse the rat sarcoma cells will then fail to multiply continuously because of the incompatibility and they will therefore disappear This was ascertained by control injection into rats No deaths have occurred as this series had been running only for 6 months

C *Rats with primary resistance towards the uninfected sarcoma* A number of rats in which transplantation of the uninfected ascites sarcoma does not take is being collected

DISCUSSION

The tumours reported here developed in rats in which a Yoshida ascites sarcoma infected by *Nosema cuniculi* had developed and regressed. After a latent period of an average of 18 months 37 per cent died with intra abdominal tumours which histologically could be classified as one group.

The classification of the tumours is based on the similarity to muscle tissue but it was not equally pronounced in all cases as for instance cross striation was found in only three cases. The degree of anaplasia varied and many parts with no resemblance to muscle tissue were therefore found. In the less differentiated and cellular parts spindle cells without resemblance to any specific mesenchymal cell dominated; such areas however were also found in the tumours in which well differentiated muscle tissue was observed. Thus in the three cases presenting spindle cells exclusively the tumours were classifed as anaplastic rhabdomyosarcomas.

Although the control series of untreated rats at present is small only, the fact that no malignant tumours have arisen in this group of 20 rats makes "spontaneous" occurrence in the strain of rats used improbable. According to the experience gained by authors who have published reports concerning the tumour incidence in various, even closely related strains of rats (*Bulloch & Curtis* 1930, *Curtis et al* 1931, *Ratcliffe* 1940, *Saxton et al* 1948, *Davis et al* 1956, *Crain* 1958, *Gilbert & Gilman* 1958, *Thompson et al* 1961) differences between strains are considerable and consequently data of tumour incidences in rats in general would be of no value for a specific strain such as the one discussed here.

As regards the possible origin of the whole group two cases deserve special mention as they developed after a shorter period than the rest, 5 and 8 monts. Areas of spindle shaped cells were found to alternate with areas of cells resembling those of a solid Yoshida sarcoma, which have not been found in the tumours with longer latent periods (Fig. 9). The solid Yoshida sarcoma is seen in Fig. 10. It is produced by subcutaneous injection of ascites sarcoma cells and appears as a uniform aggregation of the type of cells seen in an ascites smear (Fig 11). Spindle-shaped cells or still more highly differentiated cells have not been seen in a number of sections from 10 tumours of this type.

The differences between the nosema infected and the non infected sarcoma have been described by the author in a previous communication (*Petri* 1966). It was mentioned that survivors in the group of rats with the infected sarcoma may harbour viable sarcoma cells for periods far beyond the expected survival time, while such survivors have never been observed among several thousand passages of the non infected sarcoma.

Three such cases have been observed in which malignant cells could

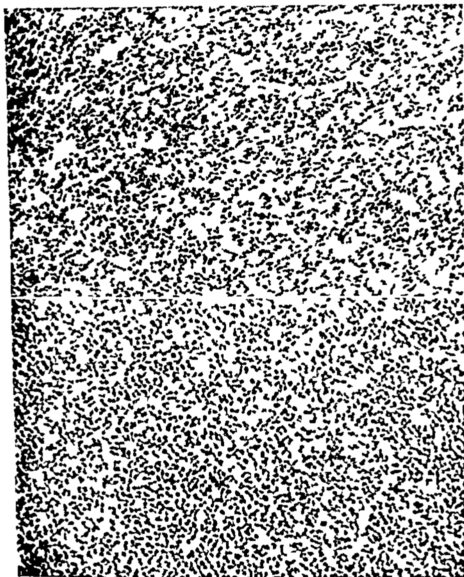


Fig 9 Area resembling the solid Yoshida sarcoma Haematoxylin erythrosin 220 ×
Fig 10 Yoshida sarcoma Solid subcutaneous tumour Haematoxylin erythrosin 220 ×

be found in the peritoneal cavity until six months after transplantation The ascitic tumour then regressed and the animals continued in apparently good health Shortly afterwards they were killed and neither fluid nor solid tumour tissue was found at autopsy It is not known however whether undiagnosed malignant tissue occasionally may lie dormant at other sites even beyond the time of regression of the ascitic

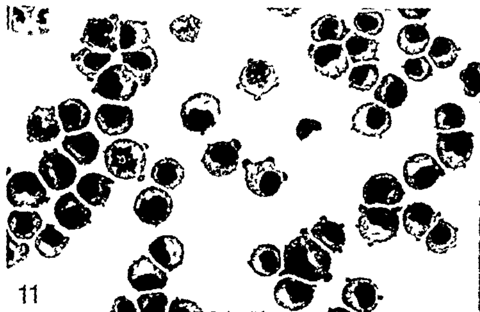


Fig. 11. Yoshida sarcoma. Dry smear of ascitic tumour. Giemsa. 440 ×

tumour. But if this were the case in the rats with late tumours it will be necessary to postulate that the Yoshida sarcoma cell—which is presumably a malignant monocyte (see Fig. 11)—must have changed, perhaps involving dedifferentiation and subsequent differentiation towards a muscle cell type. If so a further "activation" must occur to make the tumour manifest. The finding of a solid tumour 5 months after inoculation of the nosema infected sarcoma in which spindle shaped cells had developed though otherwise it retained some similarity to a solid Yoshida sarcoma, may suggest a change of cell type. The sarcoma found after 8 months also had such areas while the rest had not.

Otherwise there is, however, little experimental evidence in support of this possibility. Transplantation of spleen and liver tissue from survivors to healthy rats have been performed during the silent interval though only in three cases, to see whether viable sarcoma cells were present, but results were negative. Further, in two myosarcoma cases resistance towards the growth of the Yoshida sarcoma had previously been demonstrated in that subcutaneous injection of ascites sarcoma failed to produce a solid tumour. It is known that rats surviving inoculation of the infected sarcoma are resistant in this way (*Petri* 1966).

The question whether *Nosema cuniculi* may have a carcinogenic effect cannot be answered at present as this control series had been running only for a short time when the present manuscript was written. Other agents, possibly viral, inside the parasite or sarcoma may be involved, but a series of electronmicrographs (*Petri & Schiødt* 1966, *Petri* unpublished) failed to provide evidence.

It remains that the occurrence of 37 per cent of sarcomas of obvi-

ously myogenic origin is associated in some way with the inoculation growth and regression of nosema infected ascites sarcoma The occasionally observed equilibrium between infected ascites sarcoma and host animal and the histological appearance of the earliest of the late tumours may be suggestive of a late recurrence of the Yoshida sarcoma though of different histology This is however only offered as a tentative explanation Further in 10 survivors which were not left to die spontaneously signs of tumour formation were not observed at autopsy so that the silent interval involved in this assumption remains unexplained

SUMMARY

1 Transplantation of the Yoshida rat ascites sarcoma carries a mortality of over 95 per cent In rats inoculated with a sample of this tumour infected by the intracellular microsporidian parasite *Nosema cuniculi* (known previously as *Encephalitozoon cuniculi*) the mortality is 86 per cent

2 Among a total of 80 rats *i e* 14 per cent which survived transplantation of the infected sarcoma 43 were kept until they died upon which they were autopsied death occured after an average of 18 1 months after transplantation An incidence of 37 per cent of intraperitoneal rhabdomyosarcomas was found in these

3 The tumours were all located to the fibrous scar tissue which is found between the agglutinated intestinal loops after regression of the nosema infected ascites sarcoma

Out of the 16 sarcomas found 11 could be classified as rhabdomyosarcomas of varying degrees of anaplasia while 5 did not contain undubious myogenic cells Because of the histological similarities of the two groups groups of tumours and their common origin and macroscopic appearance they are regarded as an entity

4 The solid form of the Yoshida sarcoma is a uniform aggregation of rounded monocyte like cells and thus it is histologically different from the late sarcomas The two earliest of these however displayed some similarity to the Yoshida sarcoma and it is therefore possible that a change of cell type has taken place Further prolonged persistence of ascitic tumour cells has been observed in other rats inoculated with the parasitized sarcoma Therefore the late tumours may possibly be late recurrences of the original tumour

5 In a control group of 20 rats which were kept untreated until they died at an average age of 2 years and 6 months no sarcomas developed

6 At present a carcinogenic effect of *Nosema cuniculi* cannot be excluded

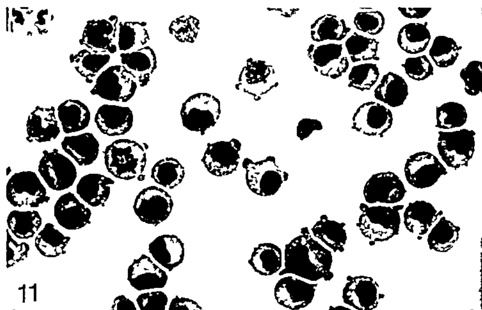


Fig 11 Yoshida sarcoma Dry smear of ascitic tumour Giemsa 440 ×

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THE COLD HAEMAGGLUTININ SYNDROME AND CARCINOMA OF THE PROSTATE

A Case Report

By

J Soltoft and K Lind[1]

Received 1 ix 67

The cold haemagglutinin syndrome (CAS) is a rare chronic disease characterized by a very high titre of cold haemagglutinins in the serum Raynaud phenomena on exposure to cold haemolytic anaemia and various degrees of cold haemoglobinuria (4 13) The γM immunoglobulins to which class the cold haemagglutinins belong (10 19) are in most cases pathologically increased but generally to a lesser degree than in Waldenstrom s macroglobulinaemia (WM) (24) These two diseases are apparently closely related The macroglobulins isolated from patients with WM have been shown to be monoclonal proteins (18 6) as have the cold haemagglutinins isolated from patients with CAS (11) *Schubothe* (24) *Waldenstrom* (26) *Kappeler et al* (12) and *Michon et al* (20) have discussed the possible connection between WM or CAS and neoplastic disease The coexistence of these conditions has been observed in several patients (4 24 20 26 2 23 21 16) but the data so far available seem to be insufficient to demonstrate any real connection WM or CAS and neoplastic disease occur predominantly in the higher age groups thus the concurrence of both might be accidental To the authors knowledge only a few autopsies of patients who have died with the CAS have been described (17 5 9 25) It was therefore of interest to collect data from patients with this disease in particular with regard to the possible coexistence of different pathological cell clones in the same patient

A patient with CAS and a metastasizing prostatic carcinoma is described

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This study was supported in part by grant from the *P Carl Petersens Fond* (K L.)
We are indebted to *B Mansa* pharmacist Department of Biophysics Statens Seruminstitut who has carried out the ultracentrifugal and immunoelectrophoretic analyses and to *H Wolthers* M D chief pathol gi t Pathological Department Vejle Amts og By Sygehus Denmark who perfo r d th po t mortem examinations

[1] Requests for reprints should be addres d t l Lin l M D Statens Se uminstitut Amager Boulevard 80 Copenhagen S Denm k

MATERIAL AND METHODS

The cold haemagglutinin titrations the antiglobulin reactions test for haemolysin analytical ultracentrifugation and immunoelectrophoresis have been described previously (14) The immunoelectrophoretic analyses were carried out using polyvalent antisera and specific antisera against light and heavy chains of γG γA and γM immunoglobulins

Case Report

An 85 year old man was admitted on November 22nd 1965 During an admission in 1962 for lobar pneumonia rectal exploration had revealed a small prostate which was not prominent there was an indefinite outgrowth from the upper right border Serum acid phosphatases were within the normal range X ray examination of the pelvis and the spine showed no evidence of metastases but a marked spondylolisthesis Since his discharge in 1962 the patient had felt well In June 1965 he experienced his first Raynaud phenomena on even slight exposure to cold He became increasingly fatigued and since September 1965 he had attacks of epigastric pain 15 days before admission the patient had a melaena

On admission the patient s complexion was very anaemic but not icteric Several lymph nodes were found in the left supraclavicular fossa On rectal exploration the prostate was found to be greatly enlarged especially the left lobe which felt solid and knotty The were no trophic changes of the extremities ears or nose

Routine Laboratory Investigations

The haemoglobin concentration which was initially 4 9 gram per cent showed a tendency to decrease despite repeated transfusions of a total of 1750 ml of packed group O erythrocytes The patient s serum contained no anti D or other irregular blood group antibodies The differential count in the peripheral blood was normal Reticulocytes 0 6 and 1 6 per cent Thrombocytes 600 000—40 000–122 000 per μl Leucocytes 7 760—4 500 per μl Serum haptoglobin 397 mg per cent (by a modification of Jayle s activation method) Tests for coagulation factors PP test was 93 per cent of normal Quick test 18 4 sec Cephalin test > 100 per cent of normal Screening test for factors VIII and IX probably not decreased Bleeding time 1 min 45 sec Alkaline phosphatases varied between 40 7 and 85 2 King Armstrong units Acid phosphatases 9 6 and 7 5 King Armstrong units Icterus index (*Meulengracht*) 2 normal Glutamic oxalic transaminases 27–73 units (*Value Wroblewsky*) Creatinine 1 0–1 5 mg per cent (Jaffes reaction Lloyd s absorption) ESR was not determined at 37 C Faeces benzidine test + → + + + but no melaena Urinalysis no glucose no albumin no Bence Jones protein microscopy + leucocytes + erythrocytes Examination of bone marrow (sternum) showed small groups of tumour cells but otherwise nothing abnormal

Special Laboratory Investigations

Cold haemagglutinins The titre two days after the first transfusion of 550 ml packed erythrocytes was 128 000 at 4 C The titres at 25 27 and 30 C were 5 120 2 560 and 640 respectively all with adult group O erythrocytes Cord blood erythrocytes of group O were agglutinated to the same titre at 4 C

Cryoglobulin The serum contained a cryoglobulin precipitating at 0 C and dissolving again at 37 C

The *Sia* test was negative

Antiglobulin test The patient s erythrocytes from blood collected in sufficient heparin to inhibit the action of complement were agglutinated by an anti non γ globulin serum

Haemolysins Both Ham s and Crosby s tests and Donath Landsteiner s test for diphasic haemolysins were negative[1] The patient s fresh acidified serum haemolysed both normal d nor group O erythrocytes and own erythrocytes at 4 C and at 37 C

On *analytical ultracentrifugation* of serum the following sedimentation coefficients and distribution of the components were calculated

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[1] The Blood Group Department Statens Seruminstitut kindly performed these tests by routine methods

	S_0	Percentage of total protein	
		Patient	(Normal values)
Albumin	3.8	69.7	(76–89)
Globulin	6.0	22.4	(9–20)
Macroglobulin	15.4	7.9	(1–5)

Immunoelectrophoretic analysis of the serum revealed a γM globulin precipitation line within a somewhat narrow mobility range. The shape and extension of the line thus deviated from normal γM lines and was similar to the γM lines generally found in patients with the CAS. The γG and γA globulin precipitation lines showed normal shape and extension and these globulins were judged to be present in normal concentrations.

X-ray examination of the bones revealed no evidence of myelomatosis. X-ray of the alimentary tract revealed a diaphragmatic hernia and the abnormal position of the ileum raised the suspicion of an intestinal or retroperitoneal tumour.

The clinical course during admission was characterized by constant occult bleeding from the intestinal tract and severe anaemia. No Raynaud phenomenon was observed and provocation was not attempted. The patient became debilitated and died of a bronchopneumonia.

Post Mortem Examination (H. Wolthers, M.D.)

In both lungs sequelae of both previous and recent bronchopneumonias were present. In the hili and adjacent to the vessels in the neck several lymph nodes (diameter 0.75 cm) were seen. In the caudal part of the oesophagus there were small erosions. The spleen was moderately enlarged. In the anal part of the sigmoid colon some broad whitish solid infiltrations were seen in a 2 cm long zone directly beneath the serosa. In the mesentheric root there were some whitish lymph nodes (up to 1 cm in diameter). Several lymph nodes (1 × 2 × 3 cm) were seen along the aorta and a single one near the portal vein. These lymph nodes were solid with a shining white cut surface. The prostate measured 4 × 5 cm, the surface was nodular and solid and on the cut surface several grey-white adenomas up to 1 cm in diameter were seen. On opening the vertebral column this was seen to be a yellow-brown colour with sloughing in some places.

Microscopical examination. The prostate contained an adenocarcinoma with extensive infiltration leaving only small amounts of normal tissue.

The wall of the colon was normal but there were tumour infiltrations in the serosa.

The spleen showed strikingly many large pyroninophilic mononuclear cells. A great number of plasma cells were seen, in places densely accumulated both in the Malpighian follicles and in the pulp. The number of eosinophilic granulocytes was also increased. These cell types were found also in a small accessory spleen.

In the para-aortic lymph nodes there was massive metastatic tumour infiltration of the same cell type as in the prostate. These metastasizing cells were also localized to some of the cervical lymph nodes which in addition showed a strikingly increased number of plasma cells. The only pathological feature in the hilar lymph nodes was an increase in plasma cells.

The bone marrow of the spine was fibrous with metastatic tumour infiltration. No other pathological cell forms were seen.

The lungs showed bronchopneumonias without evidence of tuberculous infection.

Summary of Case History

An 85 year old man was admitted because of anaemia and melaena. For 6 months preceding admission the patient had suffered from Raynaud phenomena provoked by the cold. In the hospital signs of a metastasizing prostatic cancer were found. Furthermore the demonstration of cold haemagglutinins of a very high titre, haemolysins active against the patient's erythrocytes which showed a positive Coombs

test of the non γ type and the anamnestic information of the Raynaud phenomena established the diagnosis of the cold haemagglutinin syndrome During admission there was a constant occult intestinal haemorrhage The patient deteriorated and died with a broncho pneumonia

Summary of autopsy findings Bronchopneumonia Advanced cancer of the prostate Metastatic infiltration chiefly in the bone marrow and also in some of the lymph nodes Increased number of large pyroninophilic mononuclear cells and plasma cells in the spleen and lymph nodes

DISCUSSION

Three years and six months prior to the present hospitalization the patient had been admitted because of a pneumonia which responded promptly to penicillin and sulphonamide treatment The serum was not tested for cold haemagglutinins Exploration of the rectum gave rise to a slight suspicion of a prostatic cancer From more recent knowledge however it seems likely that the patient at that time already had a malignant process in his prostate although it did not appear to be metastasizing The anamnestic information about the Raynaud phenomena suggests that the cold haemagglutin syndrome had been present for at least 6 months

The intestinal haemorrhages seem to have been an important symptom No other signs of haemorrhagic diathesis were recorded Coagulation time and bleeding time were normal as were those coagulation factors which were tested The bleeding may have originated from the tumour infiltrated part of the colon which was demonstrated at autopsy

From a pathogenetic point of view the concurrence of neoplastic disease and CAS in one patient is interesting In a series of 20 other patients with CAS representing all cases registered during the last 9 years there was one case of seminoma of the testis ((13) case 3) and one case of carcinoma of the rectum four of the remaining CAS patients suffered from WM The patients were from 55 to 90 years old (15) The establishment of a possible correlation between these diseases must await further accumulation of similar cases Among the 20 patients with CAS microscopic examination of the bone marrow had been carried out in 17 Three of these patients who also had WM showed a marked proliferation of lymphocytes in the sternal bone marrow and the fourth patient with WM had a marked proliferation of plasma cells and plasmoblasts at that site The marrow obtained by sternal puncture from the other 13 patients with CAS revealed only diffuse hyperplasia of the haemopoietic cells typical of haemolytic anaemia

Proliferation of different types of lymphoid cells in various proportions of examined bone marrows from patients with the CAS has been described (24 7 22) The question whether these cells produce cold

haemagglutinins has not been clarified Van Furth et al (8) demonstrated *in vitro* an increased synthesis of cold haemagglutinin as γM globulin from bone marrow of patients with the CAS and by means of the immunofluorescence technique they found that only the plasma cells in the bone marrow reacted with an anti γM globulin whereas the lymphoid cells which were slightly increased in number did not react Similar observations have been reported by *Curtain & Baumgarten* (3)

In the present patient the increased number of plasma cells or large pyroninophilic reticulum cells may represent the pathological cell clone responsible for the production of monoclonal cold haemagglutinins The spleen and some of the lymph nodes were heavily infiltrated by these cells but the bone marrow was not It is suggested that the pathological cells suspected to be the site of production of cold haemagglutinins should also be searched for by biopsy of lymph nodes in patients with CAS

Basing their hypothesis on the finding of various cytological forms of W M *Argani & Kipkie* (1) have proposed that the cellular origin of macroglobulins may depend on the variable factors which influence the proliferation of the reticulum cells and determine the direction of their differentiation

It seems reasonable that if such factors act in patients with macroglobulinaemia they might also act in the pathogenesis of coexisting tumour cell clones

SUMMARY

A case of a metastasizing prostatic carcinoma and cold haemagglutinin syndrome in a patient who died at the age of 85 years is presented In addition to the carcinoma cells a great number of plasma cells were found in the spleen and lymph nodes The possible relationship of these cells to the cold haemagglutinin syndrome in the patient and the occurrence of pathological cell clones in other patients with both neoplastic diseases and production of monoclonal macroglobulins are discussed

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MITOTIC ACTIVITY OF PROSTATIC EPITHELIUM

A Study by Means of Colcemid

By

IVAR LIAVÅG

Received 5 ix 67

In tissues with labile cells there is normally a continuous loss of cells and concomitantly a renewal of cells takes place tending to maintain equilibrium. This is the physiologic regeneration. According to *Bizzozero* (1894) and *Leblond & Walker* (1956) the prostate belongs to the organs with stable cells and mitoses are therefore rare. Stable cells however retain the ability to devide mitotically throughout life and following tissue loss regeneration may take place (*Bizzozero* 1894, *Cowdry* 1942, 1957).

The literature on regeneration of prostatic epithelium is meagre. It seems to have been the consensus that replacement of prostatic epithelium does not take place to any great extent. *Cameron* (1962) stated that regeneration in the prostate is limited.

To my knowledge information about the mitotic activity of human prostatic epithelium is entirely lacking. Thus mitoses have not been demonstrated in normal or in atrophic glands. Even in benign hyperplasia mitoses have not been shown (*Grinenko* 1914, *Persky & Leuchtenberger* 1957). In prostatic carcinomas mitoses are encountered rarely. It seems therefore to be impossible to demonstrate mitotic activity in routine histological sections of the prostate.

In a previous investigation (*Liavåg* in press) the relationship between atrophy, small alveolar proliferation and carcinoma was studied. It was evidenced that cell proliferation—interpreted as regeneration—took place in atrophic glands. However, mitoses could not be demonstrated. The aim of the present work was to investigate whether the active looking cell aggregations and buds of atrophic glands and the small alveolar proliferations connected with atrophic glands are dying or proliferating cells. In order to demonstrate mitoses the Colcemid method as suggested by *Evensen* (1965) has been used. Concentrations of Colcemid in tissue cultures of 10^{-8} g/ml arrest the mitoses in metaphase without producing any observable changes in the non dividing cells (*Schar et al* 1954).

For comparison the mitotic activity of epithelium in benign hyperplasia was also studied

MATERIAL AND METHODS OF EXAMINATION

The material consists of prostatic specimens from 10 consecutive and unselected patients from Surgical Department II Ullevål Hospital admitted for urinary obstruction The age of the patients ranged from 60 to 83 years with a mean age of 71 3 years After a routine preoperative examination transvesical prostatectomy (i e enucleation) was performed under general anaesthesia When the digital enucleation was finished several additional specimens were taken with scissors from the remaining prostate proper The pre and postoperative diagnoses were benign nodular hyperplasia in all cases

Four hours preoperatively the patients were injected intravenously with a solution containing 10 mg of Colcemid as recommended by *Berdal et al* (1965) No untoward effect of Colcemid was observed in any of the patients of this series According to the literature the dosage used is not harmful to the patients (*Eigsti & Dustin* 1957)

Colcemid from Ciba Basle in pure substance was prepared for injection by the hospital pharmacy in vials of 10 ml containing 1 mg of Colcemid per ml of the solution The vials were kept in a refrigerator at 4–6 C and thus the solution was protected from oxygen and light as recommended by *Figsti & Dustin* (1957) The vials were stored from 6–27 days before injection took place There was no visible change in the Colcemid solution during storage

The time interval from the injection of Colcemid to the removal of the prostatic specimen was designated the Colcemid time

At the same time as the prostatic specimen was taken a piece of skin from the margin of the abdominal incision was also taken and prepared in the same way as the prostatic specimen in order to serve as a control of the action of Colcemid as indicated by the presence of arrested metaphase figures only

The prostatic specimens were weighed immediately after removal and then immersed in a solution of 10 per cent neutralized formalin When after some hours the specimen was hard it was cut into 4 mm thick slices and further fixed in a solution containing 5 per cent mercuric chloride and 4 per cent formalin After fixation all slices were embedded in paraffin and from each slice one section was cut at 5 microns and stained with Mayer s haematoxylin and eosin (H+F)

The total number of mitoses in atrophic glands were counted in all sections and the number of mitoses were related to the weight of the specimen thus expressing mitotic activity of atrophic glands per weight unit of removed tissue For purposes of comparison the mitoses in hyperplastic glands were registered in the same way

It was assumed that the total area of the sections studied was approximately proportional to the weight of the specimen in each case

RESULTS

Mitoses in atrophic glands with flat epithelium are demonstrated in Figs 1 and 2 In the first of the two pictures it is seen that at the site of mitosis the cells appear active compared with the epithelium in the rest of the gland

Figs 3 4 and 5 demonstrate mitoses in solid aggregations of cells budding off from atrophic glands and in Fig 6 a mitosis is seen in a small acinus budding off from an atrophic gland

Mitoses also occur in foci of small alveolar proliferation (Fig 7) and among cells growing in solid strings (Fig 8)

For comparison a mitosis is shown in benign hyperplasia (Fig 9)

The results of the counting of mitoses are summarized in Table 1

Irregular glandular proliferations fulfilling the diagnostic criteria of carcinoma were not found in any of the 10 specimens Apparently normal glands were encountered in some parts of the specimens and mitoses were sometimes seen in these glands Pathological material however is not fit for the study of mitotic activity in normal glands and the present study was therefore restricted to glands with atrophic or hyperplastic epithelium

The same type of atrophic epithelium and the same occurrence of mitoses was found between the nodules of hyperplasia and in the specimens taken with scissors from the surgical capsule

Control *observations of the skin specimens showed good Colcemid* effect in the studied cases as indicated by the presence of mitoses in only arrested metaphases

Both in atrophic and hyperplastic glands the total number of mitoses increased with increasing weight of the specimen (Table 1)

TABLE 1

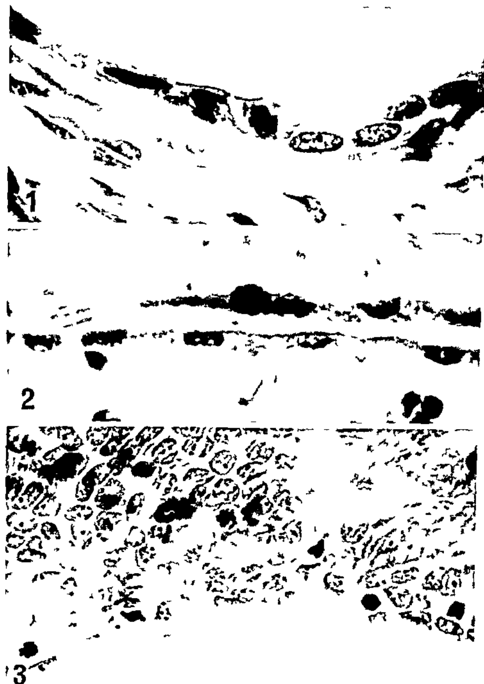
Mitotic Activity of Prostatic Epithelium in 10 Patients Operated on for Benign Hyperplasia with Urinary Obstruction

Patients		Weight of specimen (grams)	Colcemid time (hours)	Number of mitoses in atrophic glands		Number of mitoses in hyperplastic glands	
				Total	Per 10 g of tissue	Total	Per 10 g of tissue
K H	67 years	9	4½	33	42	71	79
H H	77	15	5	61	40	87	58
H A	70	17	5	127	75	143	84
L Ö	60	18	4	137	76	186	103
P P	81	25	4	201	80	272	109
V V	70	25	4½	193	80	261	104
K L	83	35	5	249	71	355	101
L I	61	40	4	303	76	405	101
O M	60	40	4	310	78	389	97
Aa S	69	50	5	356	71	512	102
Mean		27	4½	198	69	268	94

When mitotic activity was calculated per 10 g of tissue however there was no significant difference between the number of mitoses from the specimens weighing from 18 to 50 g The two smallest ones on the other hand weighing 9 g and 15 g respectively showed a mitotic activity which was also lower per weight unit than the corresponding figures for those weighing 18 g or more Definite conclusions however cannot be drawn on this point because of the small number of specimens

In Table 1 it is seen that the Colcemid time shows some variation from case to case However correction for differences in Colcemid time does not alter the conclusions mentioned above

In all specimens a greater number of mitoses were found in hyper



Figs 1–3

Fig 1 Mitosis in atrophic gland At the site of cell division the cells are large compared with the flat epithelium in the rest of the gland (No 2316/65) × 1200 H+E

Fig 2 Mitosis in a gland with flat atrophic epithelium (No 2194/65) × 1250 H+E.

Fig 3 *Four mitoses are seen in the solid aggregation of cells budding off from an* atrophic gland (No 2194/65) × 1000 H+E



Figs 4–5

Fig 4 Solid cell aggregation with one mitosis (arrow) budding off from an atrophic gland Note the globular swelling of the cytoplasm of the cell in arrested mitosis (No 2194/65) × 1?00 H+E

Fig 5 Cell aggregation with mitosis (arrow) indicates active growth in an otherwise inactive looking atrophic gland (No 275?/65) × 1?00 H+E

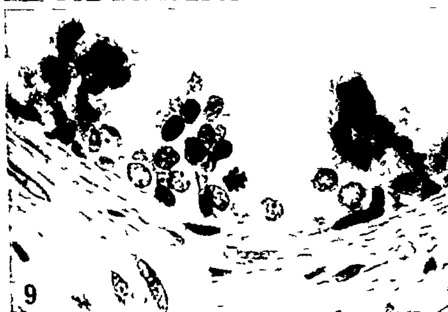


Figs 6–7

Fig 6 One mitosis is seen in a small acinus budding off from an atrophic gland (arrow) (No 2455/65) × 1200 H+E

Fig A small alveolar proliferation with two mitoses (arrows) (No 2579/65) × 1250 H+E.





Figs 8–9

Fig 8 Mitosis in an atrophic area of the pseudocapsule The epithelial cells are growing in a solid string (No 2580/65) × 1100 H+E

Fig 9 Mitosis in a gland with benign hyperplasia (No 2455/65) × 900 H+E

plastic than in atrophic glands However this is not the real picture of the mitotic activity of the two kinds of tissue because the specimens contained much more hyperplastic than atrophic glands Proportional to the glandular mass studied the mitotic activity might even be as high in atrophic as in hyperplastic glands

DISCUSSION

It was realized that a specimen which was representative for all parts of the prostate could only be obtained by total prostatectomy but in this department this method is not used in the treatment of prostatic carcinoma. In the enucleation procedure however not only the *inner gland group* is removed but even a part of the *outer gland group* or *prostate proper* which adheres to the adenomatous nodules. After the digital enucleation specimens were also taken with scissors from the remaining prostate proper. It is therefore claimed that the material represents tissue both from *the inner and the outer gland group*. With regard to histological features and mitotic activity any difference between atrophic epithelium from the central and the peripheral part of the prostate was not found.

With a view to studying mitotic activity of atrophic glands the material was also quite representative because numerous atrophic ducts and acini were found between and at the periphery of the adenomatous nodules.

In an organ as complex as the prostate a reliable standard for a quantitative estimation of mitoses was difficult to find. The glands may be closely packed or they may lie wide apart depending on the amount of fibromuscular stroma. Atrophic glands may be scattered in between the nodules of hyperplasia or form a narrow band, a wedge shaped or irregular area. The conventional method of counting mitoses in a number of microscopic fields or squares would therefore be rather unreliable. To count mitoses among a certain number of cells however might be a reliable method but very time consuming and less practical because of the relatively small number of mitoses.

By means of the Colcemid method it has been shown that there is an appreciable degree of mitotic activity in so called atrophic glands. The results of the present study makes it justifiable to conclude that the active looking cell aggregations and buds of atrophic glands and the small alveolar proliferations connected with atrophic glands are proliferating structures. The findings indicate that atrophic glands should not be looked upon only as dying structures but even as sites of regenerative growth. The question may therefore be raised whether it is justifiable to apply the term atrophic to these structures. I want to emphasize however that the definition of atrophy is based entirely on morphological criteria.

In a previous study (*Liavåg* 1967) and in the present one the question of the regeneration zones in the prostate has been studied. It was found (*Liavåg* 1967) that the small alveolar proliferation mainly took place from slit or stellate formed atrophic ducts which were deformed by periductal replacement fibrosis. Even atrophic acini sometimes seemed to be the origin of small alveolar proliferation. This accords with the observations in prostates of infants that new acini

seemed to be formed both from the acini and the ducts In the process of atrophy the acini are the first part of a lobule to disappear and regeneration takes place from the terminal branches of the ducts

In the two smallest specimens of the present series the mitotic activity per weight unit of tissue was markedly lower than in the other specimens The finding indicates that the stimulus to regeneration was reduced Since the growth of prostatic epithelium depends on androgenic hormone the finding further suggests that adrogenic stimulation was of a low level in the two smallest glands

In a previous study (*Liavag* in press) the findings indicated that atrophic glands give rise to small alveolar proliferations which in turn may develop into carcinoma This sequence is supported by the present study as indicated by the demonstration of regeneration in atrophic glands

SUMMARY

Colcemid has been used for a study of the mitotic activity in surgical specimens from patients with urinary obstruction due to benign hyperplasia Mitotic activity was found in active looking cell aggregations and buds of atrophic glands and in the small alveolar proliferations connected with atrophic glands The findings indicate that atrophic glands should not be looked upon only as dying structures but even as sites of regenerative growth

In all specimens a greater number of mitoses were found in hyperplastic than in atrophic glands However the specimens contained much more hyperplastic than atrophic glands Proportional to the glandular mass studied therefore the mitotic activity might be even as high in atrophic as in hyperplastic glands

Both in atrophic and in hyperplastic glands the number of mitoses increased with increasing weight of the specimen Per weight unit of tissue studied however there was no difference in mitotic activity of specimens weighing from 18 to 50 g On the other hand the two smallest specimens weighing 9 and 15 g showed a lower mitotic activity also per weight unit than the specimens weighing 18 g or more It is suggested that the stimulus to regeneration was insufficient or lacking in these small prostates and further that this stimulus is the androgenic hormone

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THE CAPACITY OF BREAST TISSUE TO ACCUMULATE OESTRADIOL 17β IN VITRO

By

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Since the experiments published by *Glascock & Hoekstra* (1959) several reports have appeared concerning local accumulation of oestrogens in the reproductive organs of animals following systemic administration *In vivo* studies show that these oestrogen responsive tissues exhibit a characteristic affinity for oestradiol and hexoestrol (3 4) Some *in vitro* studies have also been reported Thus *Noall & Allen* (1961) were able to demonstrate an accumulation of tritium labelled oestradiol 17β in rabbit uterus after incubation experiments *Stone & Baggett* (1965) reported accumulation of oestradiol 17β and oestrone in slices from uterus and vagina of ovariectomized mice *Terenius* (1966) found that the uptake of oestrogens by the mouse uterus *in vitro* was stereo specific in the same way as the uptake *in vivo*

The most active oestrogen oestradiol 17β was preferentially accumulated in the uterus The uptake was found to be inhibited by the respective non radioactive compound It thus seems to be well—established that the uterus and the vagina posess the ability to accumulate tritiated oestrogen following incubation

Among the oestrogens oestradiol 17β is the compound selectively accumulated by the responsive organs (4) The observation of *King et al* (1965) that oestrone incubated with slices of breast tumour from rats were metabolized in large extent to oestradiol is in keeping with this The specific uptake of oestrogens in the tissues is thought to depend on the presence of a cellular oestradiol receptor (3)

Systemic administration of tritium labelled oestradiol 17β to female rats showed that breast tissue accumulates the radioactive material (8) Accordingly cellular receptors might exist in the breast tissue as well If so a similar accumulation ability as found in uterus and vagina might be discovered when slices of breast tissue were incubated with oestradiol 17β

The present study is concerned with the possible capacity of breast

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tissue to accumulate oestradiol 17β *in vitro* Attention is also paid to the viability of the tissue slices evaluated by the tissues oxygen uptake during the incubation period

MATERIAL AND METHODS

Solutions (6 7-³H) oestradiol-17 β (specific activity 166 mC/mg) was purchased from New England Nuclear Corporation The radiochemical purity was controlled by thin layer chromatography as described previously (8) The oestradiol solution used had a radioactive purity better than 97 per cent Stock solution was prepared with 0 32 µg oestradiol per ml in 0 9 per cent NACL solution containing 5 per cent ethanol and stored at 4 C

Animals Female rats of an inbred strain were used for the experiments The body weight was from 230-275 g In order to have well proliferated breast tissue the rats were used when 16-18 days pregnant Ovariectomy and hysterectomy were performed through a small midline incision under ether anaesthesia 3 days before the start of the experiments

Incubations The animals were killed by decapitation and the breast tissue was rapidly taken out Pieces of the pectoralis major muscle were removed and used as reference in every experiment

In one of the experiments tissue from the perirenal fat capsule the vagina and the salivary gland were also used The tissues were sliced on a Stadie Riggs hand microtome and approximately 15-20 mg of tissue was put directly into the incubation flasks Each flask contained 2 ml of Krebs Ringer phosphate buffer pH 7 4 with 1 mg of glucose/ml In one of the experiments incubation was carried out without glucose in the medium The buffer was prepared from stock solutions immediately before use and tritium labelled oestradiol 17 β 8 × 10⁻³ µg was added to each flask Oxygen was applied to the surface before the flasks were closed The incubations were carried out with continuous shaking at 37 C

Measurement of radioactivity After incubation the tissue specimens were carefully blotted dry with filter paper and the wet weight was recorded The slices were put into vials containing 4 ml of ethanol After shaking the slices were left in ethanol at 4 C over night They were then washed 5 times in 2 ml of ethanol The whole ethanol fraction was brought into counting vials and taken to dryness in vacuo The residue was dissolved in 10 ml of toluene containing 0 5 per cent PPO (2 5 dephenyl oxazole) and 0 03 per cent POPOP (1 4 bis(4 methyl 5 phenyloxazol 2 yl)benzene)

The radioactivity was then measured in a liquid scintillation spectrometer (Nuclear Chicago Mark 1 model 6860)

All counts were corrected for background activity quenching and machine efficiency and transformed to disintegrations per minute All samples were counted with more than 40 per cent efficiency After ethanol extraction the tissue slices were homogenized in 2 ml of 0 5 N NAOH by ultrasonic disintegrator (MSE 100 Watt Ultra sonic Disintegrator) Aliquots were taken for protein determination (6) The amount of radioactivity left in the tissue slices after ethanol extraction was less than 3 per cent

The uptake of radioactivity in the tissue slices were expressed both in D P M per mg wet weight and as D P M per 100 µg of protein

The radioactivity in the medium was measured after ether extraction Tissue for histological examination was embedded in paraffin and sections stained with haematoxylin/eosin

Determination of oxygen uptake in slices of breast tissue during the incubation procedure The amount of oxygen taken up was measured at 37 C by Warburg technique (11) The reaction mixture was freshly prepared Krebs Ringer phosphate buffer 3 ml at pH 7 4 with and without glucose (0 5 mM or 5 mM) Breast tissue and skeletal muscle (40-50 mg) was prepared as described above Manometer readings were carried out every 15 minutes after a 10 minutes pre incubation period The incubation was stopped after 3 hours and the total protein content of each flask determined by the method of *Lowry* (6) The amount of oxygen taken up was calculated as mµ atoms O per mg of protein

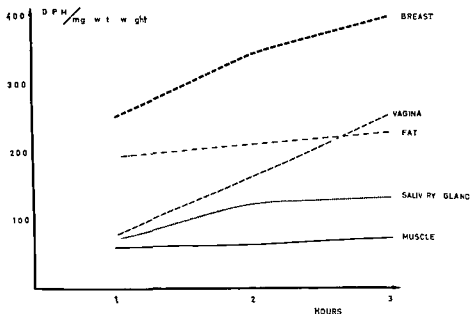


Fig 1

The concentration of radioactivity in tissue slices from various organs after incuba tion in Krebs Ringer phosphate from 1–3 hours All media contained 8×10^{-5} μg (6 7–³H) oestradiol-17β/2 ml medium Each point on the curve represent the mean value of five observations (1 2 and 3 hours incubation)

RESULTS

The concentration of radioactivity in various tissues after incubation in Krebs Ringer phosphate with tritium labelled oestradiol is shown in Fig 1 In the periods studied there is a progressive uptake of radio activity in the breast tissue There is also a progressive uptake in the vagina but the uptake does not reach the level observed in breast tissue The progressive uptake in these two tissues are in contrast to the uptake observed in skeletal muscle and fat These control tissues exhibit only slight increase in accumulated radioactivity during incu bation up to 3 hours In the salivary gland increase in uptake of radio activity occurs between 1 and 2 hours but from 2 hours up to 3 hours the level of radio activity does not increase

Protein content may not be an appropriate reference when concen tration of radioactivity in different tissues is compared The protein content in fat is very low but it is large in muscle and uterus Accord ingly concentration of radioactivity is given as D P M per mg wet weight Extraction of radioactivity with ether before and after the incubation procedure shows that from 85 to 90 per cent of the radio activity remains in the medium after incubation From 15–10 per cent of the radioactivity was recovered in the incubated tissue

The uptake of oestradiol in breast tissue and skeletal muscle was

also calculated as the ratio between concentration of radioactivity in the tissue and the medium (Table 1) It appears that breast tissue has a distinctly higher concentration of radioactivity than skeletal muscle in all the incubation periods examined In the period from 1 hours to 3 hours breast tissue is continuously accumulating radioactivity whereas radioactivity in muscle is fairly constant during the same incubation period

TABLE 1

The Uptake of Radioactively Labelled Oestradiol-17β by Breast Tissue and Skeletal Muscle after Various Incubation Periods

Incubation period	Ratio $\frac{\text{breast tissue}}{\text{medium}}$	Ratio $\frac{\text{muscle}}{\text{medium}}$	Ratio $\frac{\text{breast tissue}}{\text{muscle}}$
1 hour	18 8 ± 1 0	2 6 ± 0 2	7 4
2 hours	24 4 ± 0 6	2 7 ± 0 2	9 0
3 hours	26 9 ± 0 5	2 5 ± 0 1	10 3

Each figure represents the mean value of three observations The concentration ratios are calculated as $\frac{\text{D P M per mg wet tissue}}{\text{D P M per mg of medium}}$

Mean values and s e m (standard error of the mean) are given

The glucose concentration 1 mg/ml in the incubation medium does not significantly affect the uptake of tritiated oestradiol (Table 2)

TABLE 2

The influence of Glucose Enrichment in the Medium on the Uptake of Oestradiol-17β

Animal no	Medium with glucose	Medium without glucose
1	275 ± 10	250 ± 13
2	258 ± 13	278 ± 22
3	286 ± 19	300 ± 9
4	302 ± 18	266 ± 14
5	281 ± 21	270 ± 10

The values from five animals are given Three slices from each animal were incubated with glucose 1 mg/ml Another three slices from the same animal were incubated without glucose enrichment Values are calculated as D P M per 100 μg of protein Mean values and s e m are given

The uptake of gaseous oxygen in slices of breast tissue incubated in Krebs Ringer phosphate buffer is shown in Fig 2 There was a progressive uptake of oxygen in breast tissue and muscle during the procedure the uptake of oxygen being distinctly higher in breast tissue than in muscle The oxygen uptake in muscle was not significantly changed by the various concentrations of glucose added to the medium hence only one line is plotted In breast tissue however the oxygen uptake is distinctly higher when incubated in medium without glucose

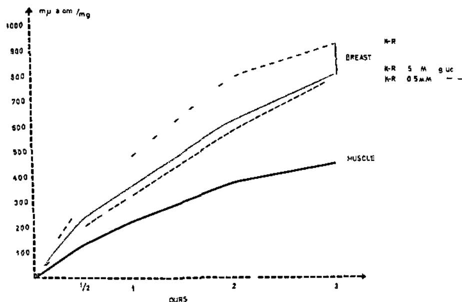


Fig 2

Uptake of oxygen in incubated slices of breast ti sue and skeletal muscle The three separate lines plotted for breast tissue show the influence of glucose added to the medium

enrichment This effect is present during the entire incubation period The reduction observed with glucose in the medium was most promi nent after 2 hours incubation less marked after 3 hours

DISCUSSION

Skeletal muscle salivary gland and fat are considered non target organs for oestradiol These tissues seem to be practically saturated with radioactivity after incubation for 1 hour with oestradiol ³H The significantly higher concentration of oestradiol ³H observed in fat com pared to muscle and salivary gland may be explained by the fact that oestradiol is fat soluble and by different physical characteristics of these tissues

The progressive accumulation of oestradiol ³H in breast tissue and vagina during the incubation period is in contrast to the findings in control tissues The uptake curve shows that breast tissue is not saturated with oestradiol ³H even after 3 hours There is however a bending of the curve after 2 hours incubation which suggests a possible limit in uptake capacity The vagina is generally accepted is a target organ for oestradiol 17β (3) In thi tud b east ti sue is found to accumulate more oestradiol per mg wet w t th t th vagina

The continuous accumulation over a l n i er d is con

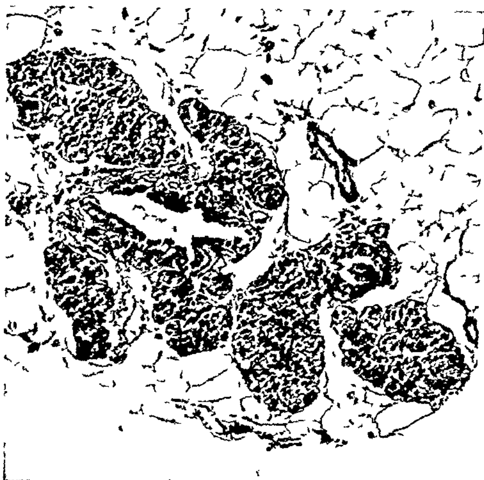


Fig 3
Breast tissue from pregnant rat with well developed ductal alveolar system H-E (× 300)

sistent with the idea that there exists also in breast tissue a receptor with the ability to catch and retain oestradiol This oestradiol binding capacity may explain the progressive uptake curve and the high concentration of oestradiol observed in breast tissue compared to the concentration in the medium

The amount of fat present in breast tissue may represent a source of error in these experiments This difficulty is partly overcome by the use of well proliferated tissue taken from pregnant rats where glandular elements are prominent in relation to fat (Fig 3) The higher accumulation and the progressive uptake of radioactivity in breast tissue is furthermore distinctly different from the findings in fat controls

The ability of tissues to accumulate oestradiol is of small interest if extensive cell necrosis occurs during the incubation period The extent

of cell necrosis is difficult to ascertain but in the present study oxygen uptake was determined as an index of viability

Breast tissue and skeletal muscle show progressive uptake of oxygen for at least 3 hours and for 2 hours the uptake curve is essentially linear The reduced uptake of oxygen observed when glucose was added to the medium Crabtree effect (12) is also consistent with viability of the slices The accumulation of oestradiol ^{3}H in breast tissue is not significantly influenced by addition of glucose to the medium En dogenous substrate is apparently present in a quantity sufficient to make enrichment unnecessary when the incubation period is restricted to 2 hours

The *in vitro* findings support the concept that normal breast tissue possesses the ability to accumulate oestradiol 17β This is in accordance with earlier *in vivo* observations of oestradiol accumulation in breast tissue of rats (8) and in human subjects (1)

SUMMARY

Following incubation with physiological levels of (6 7 ^{3}H) oestradiol 17β slices of rat breast tissue were found to concentrate radioactivity

In the incubation periods studied there was a progressive uptake of radioactivity in breast tissue A similar progressive uptake was found in slices from vagina but not in slices from skeletal muscle and fat tissue The amount of oestradiol ^{3}H accumulated was significantly higher in the breast tissue than in the control tissues

Addition of glucose to the medium did not influence the uptake of oestradiol ^{3}H in the slices The viability of the incubated slices were evaluated by their oxygen uptake

A progressive uptake of oxygen was recorded in breast tissue and muscle during the procedure

The findings are discussed in relation to the possible existence of an oestradiol receptor in breast tissue

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IMMUNOHISTOCHEMISTRY OF SARCOIDOSIS

By

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According to the investigations by *Friou* 1952 and *Sones & Israel* 1954 sarcoidosis was definitely placed in the group of diseases characterized by abnormal immune responses and most of the recent investigations on sarcoidosis have been on this immunological level. This disturbance in the immunological mechanisms is characterized by a depression in the cellular dependent delayed type hypersensitivity, while the immediate type reaction depending on circulating antibodies is quite normal or perhaps even increased (*Sands et al.* 1955). Furthermore raised and/or abnormal serum immunoglobulin levels have long been a frequent finding in sarcoid patients. However, at present no correlation between these serum globulin abnormalities and the activity of the disease has been demonstrated. It seems that patients in more chronic stages show the most constant and significant elevation in total protein and gammaglobulin fractions (*Vorberg* 1964). Investigations dealing with the different single immunoglobulins (Ig A, Ig G and Ig M) and their role in the total hypergammaglobulinaemia are highly controversial, especially regarding the more acute stages of the disease (*Patnode et al.* 1966, *Norberg* 1967 and *Daddi & Giraldroni Grassi* 1967).

There is some controversy as to the pattern of the immunoglobulins following initial immunization (primary response) and that following a booster injection (secondary response), but the most recent investigation in this field seems to show that sarcoid individuals behave according to quite normal lines (*Persellin et al.* 1966).

It is furthermore well established that anti-gammaglobulin factors frequently are present in the serum of sarcoid patients (*Kunkel et al.* 1958, *Israel et al.* 1964). These rheumatoid factors are active only against determinants in the *human* gammaglobulins, the latex test being positive and the Waaler Rose test negative. It is our impression that the latex F II test is positive in the more chronic stages of the disease, primarily among females. Apart from such rheumatoid factors

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auto immunity phenomena have not been shown in sarcoid patients (*Doniach & Roitt* 1962). However in a series of 47 sarcoid patients *Elling & Faber* (to be published) found the serum in one fourth of these to contain tissue antibodies against mitochondria. The interpretation of these findings is problematic although it shows that sarcoid individuals may exhibit some of the phenomena frequently encountered in disorders included in the group of collagen diseases.

The morphological expression of all such immunological events in the tissue has also been clearly demonstrated. Apart from the conspicuous granulomatous reaction the sarcoid tissue shows a number of other histo-morphological characteristics which appear more or less frequently. These are interesting, as they lend support to important interpretations of the pathogenetic mechanism of the disease (*Teilum* 1948 and 1964).

Such morphological characteristics are: 1) A prominent plasmoblastic proliferation in relation to the granulomatous areas, 2) the occasional finding of central areas with fibrinoid necrosis constituting and allergic granuloma and 3) a tendency to extra- and intra-granulomatous hyalinosis with final loss of histological and cellular details of the sarcoid tissue. Sometimes this hyalinization is laid down in several concentric lamellae around the granulomas and the vessels, i.e. the so called "onion skin lesion" which by now is known to occur not only in systemic lupus erythematosus but also in a number of other disorders in the group of collagen diseases.

In a morphological study *Teilum* 1948 interpreted these hyaline bands as a hyperglobulinosis locally in the tissue, possibly as a result of an antigen antibody precipitation. Actually in SLE and other disorders of mesenchymal tissue such substances were later shown, using the fluorescent antibody technique, to contain human gammaglobulin (*Vazquez & Dixon* 1957).

As sarcoidosis thus show several serological and morphological changes suggestive of a collagen disease we found it of interest to investigate whether such similarity could be extended also to apply to the immunological events in the tissues as elucidated by means of the fluorescent antibody technique.

MATERIAL AND METHODS

The material derives from 10 patients with generalized sarcoidosis. A series of different stages of granuloma formation are included in the material, i.e. morphologically young, active cases with purely cellular granulomas and more advanced stages involving increasing degrees of hyalinosis.

The tissue investigated consisted of lymph nodes from the paratracheal region. Immediately after surgical removal they were frozen in isopentane and solid carbon dioxide and stored at −70 C until investigations were performed, usually within 1-2 weeks after removal. 2 inguinal and 2 mesenterial lymph nodes showing varying degrees of non-specific reticulosis served as a control group.

Conventional paraffin embedded tissue was stained with haematoxylin-eosin, van Gieson, methylgreen-pyronine and periodic acid Schiff (PAS) staining techniques

Both the direct and the indirect immunofluorescent techniques were employed using labelled rabbit antisera against human serum components and in addition human sera containing antibodies against different tissue substances with special emphasis on the reaction of certain sera from sarcoid patients included in *Fling & Faber*s material

Rabbit antisera containing precipitating antibodies against human immunoglobulins albumin and fibrinogen were prepared by immunization of albino rabbits with Cohn fractions of pooled human donor blood Rabbit antisera containing precipitating antibodies against the separated human immunoglobulins Ig A Ig G and Ig M human complement (β_1 A/C + β_1 E) were obtained from Central Laboratory Red Cross Amsterdam

The specificity of the antisera employed was tested by immuno electrophoresis and was shown to give monospecific reactions using whole human serum and sera containing myelomproteins and macroglobulins as antigens as described earlier (*Fling 1966*) The antisera were precipitated twice with saturated $(NH_4)_2SO_4$ and the sediment dissolved in phosphate buffered saline (PBS) after dialysis against PBS they were conjugated with 25 mg of fluorescein isothiocyanate (FITC) per g of protein Free fluorochrome was removed by passage of the solution through a Sephadex G50 column Before use the conjugate was absorbed with powder of acetone dried guinea pig liver

4–5 μ thick unfixed tissue sections were cut in a cryostat and dried with a fan for 2 hours at 4 C In the *direct* technique the sections were then incubated with the different FITC conjugated antisera for 30 minutes at room temperature and washed rigorously with PBS for 30 minutes and then mounted with cover slips using PBS buffered glycerine In the *indirect technique* tissue sections were prepared as described but after drying they were incubated with human sera for 30 minutes washed with PBS and then incubated with FITC labelled rabbit anti human gamma globulin for 30 minutes After washing the slides were mounted with cover slips

The microscope was a Leitz Ortholux and Orthoplan with equipment for simultaneous light field fluorescent and phase contrast microscopy The UV light source was a high pressure mercury lamp (Osram HBO200) An UG1 exciter filter was used together with an UV absorbing barrier filter

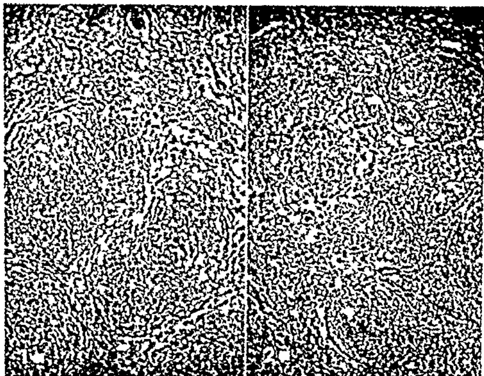
Blocking tests were performed by incubating sections of a sarcoid lymph node with non labelled antisera for 30 minutes after repeated washing with PBS the sections were reincubated with the homologous FITC labelled antiserum In all cases a total blocking of the observed specific fluorescence occurred *Absorption* of rabbit antisera against human immunoglobulins with human gammaglobulin isolated chromatografically from a normal human serum (kindly supplied by *B Mansa* Biophysical Department Statens Seruminstitut) also eliminated totally the specific fluorescence

Finally incubation of the sections with various solutions of FITC in PBS showed that the fluorochrome was not in any case fixed to specific structures in the sarcoid tissue

RESULTS

All the sarcoid lymph nodes investigated in the present study showed basically the same findings but with some quantitative differences apparently dependent on the stage of granuloma formation As the granulomas mature characteristic changes in the morphology occur these are 1) a decrease in cellularity and 2) cellular hypertrophy simultaneously with 3) an increase in the formation of fibro hyaline intra as well as extragranulomatous substances In the present study care was taken to select a material with a reasonable spectrum of these different stages of granuloma formation

Direct technique Using FITC labelled rabbit anti human gamma globulin all sarcoid lymph nodes exhibited considerable amounts of *immunoglobulins* in comparison with amounts seen in controls As a constant feature varying degrees of fluorescence were present in the



Figs 1–5

Fig 1 a Section from sarcoid lymph node showing four granulomas without hyalinization In between the granulomas a lively cellular proliferation is present in addition to sparse perigranulomatous hyalinosis Phase contrast × 200

Fig 1 b Same field as Fig 1 a viewed in the fluorescence microscope after incubation with FITC labelled rabbit anti human gammaglobulin An accumulation of immunoglobulins within the granulomas (compare phase contrast) is evident although small central areas remain unstained Also the proliferating inter granulomatous areas show marked fluorescence × 120

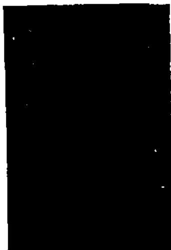
Fig 2 a Section from sarcoid lymph node showing four granulomas including one partly hyalinized granuloma Phase contrast × 200

Fig 2 b Same field as Fig 2 a viewed in the fluorescence microscope after incubation with FITC labelled rabbit anti human gammaglobulin Varying degrees of staining of the three cellular granulomas intense staining of the hyaline substances in and around the fourth × 120

Figs 3 and 4 Sections from sarcoid lymph nodes showing several granulomas some of which partly hyalinized Viewed in the fluorescence microscope after incubation with FITC labelled anti human β_{1C} globulin (third part of complement) intensive staining of the hyaline substances localized within and in between the granulomas is evident Furthermore a weaker fluorescence of some cellular granulomas is seen × 120

Fig a Section from sarcoid lymph node stained with haematoxylin-eosin and viewed in UV light Selective fluorescence of the hyaline substances situated in between around and within the granulomas × 120

Fig b Section adjacent to that depicted in Fig 5 a stained with FITC labelled anti human β_{1C} globulin (complement) Intense fluorescence localized to the hyaline materials only (compare Fig 5 a) × 120



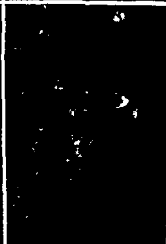
1b



2



3



4



5a



5

cell rich inter granulomatous and perivascular areas but with an accentuation in the peri granulomatous zones with a lively plasmoblastic and pyroninophilic cellular reaction This accentuation of the fluorescence was most conspicuous in the early cellular stages of granuloma formation with little or no hyalinosis The granulomas however also showed signs of immunoglobulin content Often a dark non reactive area in the centre was seen but this was clearly of a smaller size than the granuloma otherwise seen by phase contrast or ordinary microscopy (Fig 1) Thus the granulomas show accumulation of immunoglobulins which gradually mix with the highly reacting peri granulomatous areas However the intensity of this specific granulomatous fluorescence varied as some granulomas with reaction throughout the whole area were also observed in most of the lymph nodes Fig 2 illustrates such different stages of granuloma formation The field contains at least four granulomas one of these being partly hyalinized This hyaline material mainly situated in the periphery of the granuloma shows a distinctly pronounced fluorescence Such signs of the presence of immunoglobulins were also found in the hyaline dots or bands frequently present in the inter granulomatous and perivascular zones

Investigations with FITC labelled *specific immunoglobulins (Ig A Ig G Ig M)* showed that these were present in all of the sarcoid lymph nodes although in highly varying amounts Although quantitative determination of the immunoglobulin content in tissue sections is problematic it is our impression that Ig A and Ig M were responsible for the main part of the immunoglobulin reactivity possibly with Ig A as the most intensively reacting single component while Ig G constantly was present only in small or very small amounts

Complement The binding of β_1C globulin (a part of the third component of complement) in the sarcoid tissue was localized to the same sites as the immunoglobulins *i e* to areas in which the morphology was altered However some qualitative differences occurred Commonly the young pure cellular granulomas did not show any tendency to bind complement as a rule these appeared as dark non reacting foci As the hyalinization increased the granulomas and the inter granulo

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Figs 6–7

Fig 6 Section from sarcoid lymph node containing several cellular granulomas Staining of mitochondria using indirect immunofluorescent technique and sarcoid sera containing antibodies against mitochondria Intense fluorescence of the granulomas localized to the cytoplasm of the epithelioid cells In addition a bright fluorescence of single large cells in close relation to the hyaline substance in between the granulomas is present × 350

Fig Section from sarcoid lymph node Nuclear staining using indirect immunofluorescent technique and sera containing antinuclear factors Several granulomas in lymphoid tissue are clearly seen No fluorescence of the hyaline substances × 350


6


7

matous hyaline bands showed increasing amounts of fixed complement Although complement was also detected in small amounts in the perivascular regions and in some cellular constituents of the granulomas it seems as if *this component of complement predominantly* was fixed to the areas of hyalinosis (Figs 3–5)

Using FITC labelled antiserum against fibrinogen, fluorescence was seen occasionally in a thin linear line around the granulomas whereas some large granulomas or clusters of granulomas showed central areas with a rather strong staining The phase contrast picture of these central zones appeared as granular areas with loss of cellular details Thus these structures probably represent areas of fibrinoid necrosis The hyalinized substances were non reactive

Investigations using labelled *anti human albumin* showed a weak diffuse fluorescence of the whole gland identical in the controls and *the sarcoid lymph nodes* as expected *when a simple inhibition with* serum is responsible for the reaction pattern

Indirect technique The tissue was incubated with serum from sarcoid patients it had been shown to contain mitochondric antibodies (*Elling & Faber* to be published) The binding of these antibodies to structures in the lymph nodes was then visualized by employing a FITC labelled anti human gammaglobulin as the second layer after the serum In comparison with the control lymph nodes all the sarcoid nodes showed a strongly increased fluorescence The granulomas possessed the strongest reaction especially in single large cells including the giant cell systems The fluorescence was clearly localized to the *cytoplasm in a granular arrangement and distributed evenly* all over the granuloma (Fig 6) The hyaline areas appeared as dark non *reactive structures although large highly stained cells were seen in* close relation to these substances

Finally the sarcoid lymph nodes were incubated with a conjugated serum containing *anti nuclear factors* This technique did not offer more information than ordinary nuclear stainings The granulomas appeared only slightly stained in the preparations as they were relatively poor in nuclei in comparison with the surrounding lymphatic tissue (Fig 7) In high magnification the nuclear fluorescence was clearly ring shaped corresponding to the vesicular nuclei of the epithelioid cells The hyaline substances were non reactive

DISCUSSION

*Sarcoidosis is a systemic granulomatous disorder affecting the mesen*chymal or reticular tissue Apart from the epithelioid cell granulomas some morphological characteristics are *often present 1) A pronounced* basophilic (plasmoblastic) cellular proliferation 2) vascular involvement and 3) fibrinoid and hyaline changes also seen in *other disorders* of the mesenchymal tissue This similarity to the group of collagen

diseases is especially underlined in investigations by *Teilum* 1948 and 1964 and in the present immunohistochemical study

All of the sarcoid lymph nodes in the present study showed in principle identical findings These were

1) A constant increase in the immunoglobulin content in comparison with that of control lymph nodes

2) These immunoglobulins were accumulated in areas in which the morphology was altered As shown by *Mellors et al* 1957 and confirmed by us the proliferating perigranulomatous zones showed a bright fluorescence with anti immunoglobulin conjugates but contrary to these authors we found the granulomas proper also to contain immunoglobulins This was most conspicuous in the early active cases involving basophilic cellular proliferation and pure cellular granulomas with only little or no hyalinosis Furthermore on this immunological level the granulomas appeared to be much more differentiated as granulomas inside one and the same lymph node even inside one and the same field of vision were seen to differ considerably in extension and intensity of fluorescence This may indicate different functional stages of granuloma formation an impression also achieved by ordinary microscopy

3) In addition the hyalinosis appeared as an immunologically relevant material constantly showing signs also of an immunoglobulin content All of the single specific immunoglobulins (Ig A Ig G Ig M) were present in the sarcoid lymph nodes investigated although in highly varying amounts On the basis of the present study any correlation could not be established between stage of granuloma formation (disease activity) and pattern of these specific immunoglobulins in the tissue Establishment of such a correlation has been attempted in some *serological* investigations but the results are controversial (*Patnode et al* 1966 *Norberg* 1967 *Daddi & Giraldroni Grassi* 1967) It seems however as if Ig A most often was responsible for the main part of the immunoglobulin content in the tissue obtained from the present material

4) The complement fixing ability of the sarcoid tissue also showed a characteristic pattern In a study of the localization of complement in tissue *Lachman et al* 1962 stressed that such factors could be bound either to antigen antibody complexes or to gammaglobulin aggregates which may be trapped mechanically or be fixed by immune adherance in the tissue Although experiments ruled out the possibility that fixation of complement *in vitro* may be due to freezing and thawing of the tissue the exact significance of the binding of complement in tissue should be interpreted with some caution Making these reservations however the distinct ability of specific structures in the sarcoid tissue in particular the hyaline substances to bind complement and the failure of any comparable structure in the control lymph nodes to behave in a similar way may indicate that we are dealing either with

a specific antigen antibody precipitation or with gammaglobulin aggregates Thus there should be no reason to believe that the fibrohyalinosis in sarcoidosis represents a simple healing by scar tissue but rather they should be considered to be immunologically relevant materials which are precipitated in the tissue This would be in agreement also with the interpretations advocated by *Teilum* 1948 who on a morphological basis termed such substances an allergic hyperglobulinosis in the tissue

Thus also on the immunohistochemical level sarcoidosis has shown a number of reactions identical to those of disorders involved in the collagen diseases (systemic lupus erythematosus panarteritis nodosa etc)

5) In an attempt to characterize further the sarcoid tissue reaction a histochemical demonstration of mitochondria was performed employing a two layer immunofluorescent technique and human sera derived from patients with sarcoidosis and biliary cirrhosis By way of cell fractionation studies and by their reaction with subcellular cytoplasmic substances in other tissues these sera have been shown to contain antibodies against a mitochondrial constituent (*Elling & Faber* to be published) The fluorescence pattern obtained by these sera with all of the sarcoid lymph nodes investigated indicates that the cellular constituents of the sarcoid tissue by far are more rich in mitochondria than those of the control tissue Especially the granulomas proper are composed of cells showing prominent fluorescence indicating a presence of many mitochondria This is in agreement with some ultrastructural investigations according to which epithelioid cells among other features are characterized by an abundance of mitochondria which possibly are modified and/or degenerated (*Wanstrup & Christensen* 1966 *Basset et al* 1967 *Wanstrup* 1967) These modified mitochondria could be the antigen that might be responsible for the antimitochondria activity of sera from some sarcoid patients

Cells situated in intimate relation with the hyaline deposits also showed a prominent fluorescence with anti mitochondria sera Thus from a immunohistochemically point of view cells with signs of a highly metabolic activity are constantly connected with the basic changes in the sarcoid tissue reaction *i e* the granuloma formation and the hyaline precipitation

SUMMARY

Lymph nodes from 10 cases of generalized sarcoidosis have been investigated by the fluorescent antibody technique

The direct technique was employed using labelled rabbit antisera against human gammaglobulins (Ig A Ig G Ig M) complement fibrinogen and albumin

The material comprises a wide spectrum of morphological stages of

the sarcoid tissue reaction i e young active cases with cellular granulomas and more advanced ones with increasing degrees of hyalinization

All the sarcoid lymph nodes showed basically the same findings In comparison with the controls the sarcoid nodes exhibited a far more pronounced content of immunoglobulins These were accumulated in areas in which the morphology was altered i e in the proliferating perigranulomatous and perivascular zones and within the granulomas proper The fluorescence of the granulomas varied in extension and intensity indicating different functional stages of granuloma formation Gammaglobulins of all of the three human classes were found to be present in the tissue but no safe correlation between the presence of any of these gammaglobulins and disease activity could be established

β_{1C} globulin (a part of the third component of complement) was localized to the sarcoid tissue at the same sites as the gammaglobulins Commonly however the young cellular granulomas showed only little or no complement fixing ability while increasing amounts of complement were bound in the tissues accordingly as the hyalinization proceeded

FITC labelled antisera against albumin did not stain any specific structures while FITC labelled antisera against fibrinogen stained areas with fibrinoid necrosis which occasionally might be present in the granulomas

On the basis of this immunohistochemical point of view it is concluded that there are some reasons to accept sarcoidosis as a condition which has essential basic features in common with the group of collagen diseases Furthermore a number of histo morphological changes pointing in the same direction are stressed

Finally the indirect technique was employed using sera from sarcoid patients which had proved to contain antibodies against mitochondrial constituents The granulomas proper and cells in close relation to the hyaline material in between the granulomas showed constantly a bright fluorescence indicating an abundance of mitochondria in these cells This is in agreement with some ultrastructural findings and shows that cells with signs of a high metabolic activity are closely related to the granuloma formation and the precipitation of the hyaline substances

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FOCAL AND DIFFUSE THYROIDITIS AND THYROID ANTIBODIES

Immunopathological Correlations

By

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JOHN WANSTRUP and OLE WERDELIN

Received 26 ix 67

Antibodies against one or several thyroid specific antigens can be demonstrated in practically all sera from patients with diffuse chronic thyroiditis of Hashimoto s type (*Doniach & Roitt* 1964a) Thyroid antibodies occur almost as frequently in sera from goiterous as well as non goiterous patients with acquired myxoedema as in sera from patients with Hashimoto s disease probably because chronic thyroiditis is the most common cause of myxoedema (*Owen & Smart* 1958 *Sclare* 1963 *Doniach & Roitt* 1964a) Thyroid antibodies may also be demonstrated in about 80 per cent of sera from patients with thyrotoxicosis (*Hjort* 1963 *Doniach & Roitt* 1964a) and not infrequently in sera from patients with atoxic colloid or adenomatous goitres (*Doniach & Roitt* 1964a) thyroid carcinoma (*Stuart & Allen* 1958 *Fulthorpe et al* 1961 *Doniach & Roitt* 1964a) and even in sera from individuals without clinical signs of thyroid disease (*Goudie et al* 1959 *Hackett et al* 1960 *Hill* 1961) The existence of thyroid antibodies in sera from patients without Hashimoto s disease may reflect the occurrence of asymptomatic focal thyroiditis (*Goudie et al* 1959 *Bastenie et al* 1967) which differs only quantitatively from the diffuse chronic thyroiditis described by Hashimoto (*Hashimoto* 1912 *Simmonds* 1913 *Bastenie* 1944 *Whitesell & Black* 1949 *Williams & Doniach* 1961 *Senhauser* 1964)

This study was designed with a view to finding if possible a correlation between the occurrence of three different thyroid antibodies and the existence of chronic thyroiditis the latter term comprising focal as well as diffuse lesions



Figs 1–2

Fig 1 Chronic thyroiditis with epithelial changes (Askanazy cells)
Fig 2 Diffuse chronic thyroiditis (Hashimoto's disease) (35 ×)

MATERIALS AND METHODS

Systematic histological examinations were made of thyroid tissue from 477 consecutive autopsies without clinical signs of thyroid disease 1162 sera from individuals without overt signs of thyroid disorders were examined for the occurrence

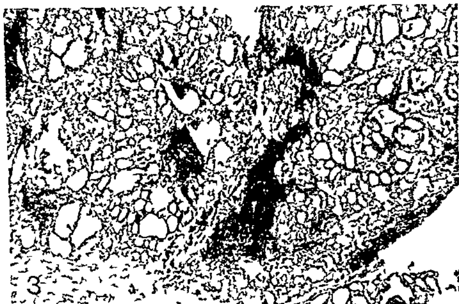


Fig 3

Focal thyroiditis (35 ×)

of three different thyroid antibodies Five hundred and sixty three subjects were examined both serologically and histologically sera as well as thyroid tissue being available in these cases This material consisted of 207 autopsies the corresponding sera having been collected from the blood bank and of 356 thyroidectomized patients from whom sera as well as surgical specimens were available Among these patients 144 had toxic goitres 159 had atoxic adenomatous or colloid goitres 32 had thyroid carcinoma and 21 had diffuse thyroiditis In order that ample tissue should be available in each case biopsy specimens were not included in this study In the autopsy series one or two were not included in this stud In the autopsy series one or two longitudinal sections from one lateral thyroid lobe were available for histological study In the series of thyroidectomies the number of sections differed widely in most cases however between two and four specimens were examined The histological sections were examined by five pathologists who were ignorant of the results of the serological examinations Thyroiditis as defined in this study includes focal or diffuse lesions in the thyroid consisting of interstitial lymphocyte and plasma cell infiltration and Askanazy cell metaplasia of the thyroid epithelium (Fig 1) These lesions may be diffuse sparing no part of the thyroid tissue a condition identical with Hashimoto s disease or they may be patchy i e foci of thyroiditis surrounded by unaffected thyroid parenchyma An attempt was made to grade the thyroid lesions in arbitrary groups 1+ 4+ according to the size and number of the foci 1+ denotes the occurrence of a few foci 2+ denotes that foci was found in many fields of vision although not in all 3+ that every low power field contained foci surrounded by broad or narrow zones of normal parenchyma and 4+ that lesions were diffuse and no normal parenchyma was left Fig 2 shows an example of 4+ thyroiditis i e diffuse thyroiditis and Fig 3 shows 2+ thyroiditis

Thyroglobulin antibodies were demonstrated by means of passive haemagglutination reaction using commercially available thyroglobulin sensitized sheep cells (Bourroughs Wellcome & Co) All sera were screened for the occurrence of the microsomal thyroid antibody by means of an immunofluorescence technique using unfixed cryostate sections of thyroid tissue and fluorescein tagged anti human globulin from Progressive Laboratories Baltimore Md U S (*Holborrow et al* 1959) Positive sera were titrated by means of a complement fixation using doubling stand

ardized microsome fractions of extracts of surgical specimens of toxic goitres as antigen (*Roitt & Doniach* 1958) CA-2 antibody was demonstrated by means of an immunofluorescence technique using methanol fixed sections of thyroid tissue (*Balfour et al* 1961)

RESULTS

Table 1 shows that thyroiditis was found in 73 (15 per cent) out of 477 thyroids examined at consecutive post mortem of patients without clinical signs of thyroid disease The incidence of thyroiditis is nearly twice as high in females as in males increasing with advancing age thyroiditis is found in about one fourth or one third of females over the age of 65 years

TABLE 1

Occurrence of Thyroiditis Lesions in 477 Consecutive Autopsies without Clinical Signs of Thyroid Disease in Relation to Age and Sex

	Number of autopsies	Number of thyroids with thyroiditis lesions	Incidence per cent
Under 40 years	68	5	7
40 – 60 years	174	25	14
Over 60 years	235	43	18
Total	477	73	
Males	303	35	12
Females	174	38	22
Total	477	73	
Females over 65 years	67	19	28

Table 2 shows that one or more of three thyroid antibodies were found in 207 (18 per cent) sera from 1162 individuals without clinical signs of thyroid disease The incidence of thyroid antibodies is three times higher in females than in males and it increases with advancing age so that almost one third of sera from females over 65 years contains one or several thyroid antibodies There is a close similarity between the incidence and the sex and age prevalence of thyroid antibodies and thyroiditis

Table 3 shows that this pattern of sex and age distribution of thyroid antibodies and thyroiditis cannot be found in the surgical groups of patients with thyrotoxicosis and atoxic adenomatous or colloid goitres

Table 4 shows the serological and histological findings in 563 patients from whom thyroid tissue as well as sera were available In patients with thyroiditis the sera are found to contain thyroid antibodies at a rate significantly higher than the one to be expected if thyroiditis and thyroid antibodies occurred by chance in the same patients On

the other hand sera from patients in whom the thyroids were infiltrated by lymphocytes which were not in close contact with the thyroid parenchyma i e absence of Askanazy cells did not contain thyroid antibodies more often than could be expected in the present material is a chance occurrence

TABLE 2

Occurrence of One or Several Thyroid Antibodies in Sera from 1162 Subjects without Clinical Signs of Thyroid Disease in Relation to Age and Sex

	Number of sera	Number of sera containing one or several antibodies	Incidence per cent
Under 40 years	173	20	12
40 - 60 years	639	117	18
Over 60 years	350	70	20
Total	1162	207	
Males	608	55	9
Females	554	152	27
Total	1162	207	
Females over 65 years	129	40	31

TABLE 3

Occurrence of Thyroiditis Lesions and One or Several Thyroid Antibodies in Thyroidectomized Patients with Toxic Goitres and Atoxic Adenomatous or Colloid Goitres in Relation to Sex and Age

	Patients with toxic goitres	Number with thyroiditis lesions	Number with thyroid antibodies	Patients with atoxic goitres	Number with thyroiditis lesions	Number with thyroid antibodies
Under 40 years	78	65 (83%)	60 (77%)	4	21 (47%)	12 (27%)
40 - 60 years	58	49 (84%)	45 (78%)	77	36 (47%)	23 (30%)
Over 60 years	8	6	5	37	15 (41%)	14 (38%)
Male	15	13 (87%)	13 (87%)	19	9 (47%)	5 (26%)
Females	129	107 (83%)	98 (76%)	140	63 (45%)	44 (31%)

Table 5 shows that agreement between serological and histological findings was found in 486 out of 563 cases (86 per cent) The divergencies are equally divided between cases with thyroiditis without thyroid antibodies and cases without thyroiditis but with thyroid antibodies The divergencies therefore cannot be explained as a conse

quence of differences between the sensitivity of the serological and the sensitivity of the histological examinations

Table 6 shows a specification of the antibody findings each subgroup being correlated to the histological findings. As seen from the table the isolated findings of the thyroglobulin antibody in titre 250 or less cannot be correlated to the existence of thyroiditis. The same is true of the CA 2 antibody when this antibody is found alone. On the other hand correlation between the isolated occurrence of even low titres of the microsomal antibody and the existence of thyroiditis was found to be good. Practically all sera containing the thyroglobulin antibody in titre 2500 or more and microsomal antibody demonstrable by the complement fixation reaction as well as almost all sera containing more than one thyroid antibody originated from subjects in whom histologically demonstrable signs of thyroiditis were manifest.

TABLE 4

Occurrence of Thyroiditis Lesions and Capsular Lymphocyte Infiltrates without Askanazy Cells Correlated to the Presence of Thyroid Antibodies

	Number of patients	Thyroid antibodies present	Incidence per cent
Number of patients	563	218	39
Patients with thyroiditis lesions	221	181	82
Patients with capsular lymphocyte infiltrates	38	15	39

Level of significance $p < 0.001$

TABLE 5

Occurrence of Thyroiditis and Thyroid Antibodies in 563 Thyroidectomized Patients and Autopsies

	Thyroiditis present	Thyroiditis absent
Thyroid antibodies present	181 (32%)	37 (7%)
Thyroid antibodies absent	40 (7%)	305 (54%)

Table 7 is identical with Table 5 following correction for antibody findings not correlated to thyroiditis. In Table 6 the isolated findings of CA-2 antibody and thyroglobulin antibody in titre 250 or less are considered negative serology. After this manipulation agreement is found between serological and histological findings in 491 out of 563 cases (87 per cent) and by far the greatest number of divergencies is due to the existence of thyroiditis in patients without antibodies in their sera. Most of the divergencies can now be explained as a consequence of the histological examination being more sensitive than the serological examination.

TABLE 6

Specific Thyroid Antibody Findings Correlated to the Presence of Thyroiditis

	Number of patients	Thyroiditis lesions present	Incidence per cent
Total material	563	[illegible]1	39
Thyroglobulin antibody alone titre 5 – 2	[illegible]	[illegible]	24
Thyroglobulin antibody alone titre 250	11	8†	73
Thyroglobulin antibody alone titre ≧ [illegible] 500	17	1[illegible]	94
Microsomal antibody alone positive immunofluorescence negative complement fixation reaction	25	19§	76
Microsomal antibody alone positive complement fixation reaction	15	15§	100
CA-[illegible] antibody alone	17	11	[illegible]5
More than one thyroid antibody	115	113	98

Levels of significance no significance
† $p < 0.05$
§ $p < 0.001$

TABLE 7

Occurrence of Thyroiditis and Thyroid Antibodies in [illegible]3 Thyroidectomized Patients and Autopsies Following Correction for Antibody Findings without Correlation to the Presence of Thyroiditis (see text)

	Thyroiditis present	Thyroiditis absent
Thyroid antibodies present	157 (28%)	9 (2)
Thyroid antibodies absent	[illegible]3 (10%)	334 (60%)

TABLE 8

High Titres of Thyroid Antibodies Correlated to Extensive Thyroiditis in [illegible]1 Cases with Thyroiditis and Thyroid Antibodies

	Total number	Extensive thyroiditis (3 and 4 +)	Incidence per cent
Total material	[illegible]1	55	[illegible]
High titre complement fixing antibody (= 64)	38	[illegible]3	60
High titre thyroglobulin antibody (= 250 000)	[illegible]0	11*	55

Levels of significance † $p < 0.01$
$p < 0.001$

Table 8 includes all cases in whom both thyroiditis and thyroid antibodies were found The table shows that high titres of microsomal antibody ($\geq$ 64) and thyroglobulin antibody ($\geq$ 250 000) are found significantly more often in sera from patients with extensive thyroiditis (3+ and 4+) than could be expected if high antibody titres and extensive thyroiditis occurred by chance in the same individuals

DISCUSSION

Among the examined post mortem cases in which clinical signs of thyroid disease were absent foci of thyroiditis were found in 15 per cent of the cases Thyroiditis occurred more often in women than in men and more frequently with advancing age A similar sex and age distribution has been described by *Simmonds* (1913) who examined one thousand macroscopically normal thyroids Thyroiditis was never seen in thyroids from patients below the age of 30 years but in 15 per cent of women and in 3 per cent of men above that age A similar sex and age distribution was demonstrated by *Goudie et al* (1959) *Williams & Doniach* (1961) *Weaver et al* (1966) and *Bastenie et al* (1967) but the over all incidence of thyroiditis found by these workers was higher than the one reported by *Simmonds* and their figures agree with the findings presented here

In the present material comprising individuals without clinical signs of thyroid disorders the incidence and the sex and age distribution of one or several of three thyroid antibodies corresponded closely to the incidence of thyroiditis in the post mortem material A similar pattern of the occurrence of thyroid antibodies in subjects without thyroid disorders has been described by *Hackel et al* (1960) *Hill* (1961) and *Bastenie et al* (1967) who examined sera from their materials for the presence of the thyroglobulin antibody only by *Goudie et al* (1959) who demonstrated the microsomal antibody only and by *Doniach & Roitt* (1964b) who examined sera from their material for the presence of three different thyroid antibodies

The incidence of thyroiditis and of thyroid antibodies in the present surgical material of patients with pathological thyroids is higher than the incidence in the two materials previously mentioned in this paper and in contrast to these both sexes and all ages are equally represented among the patients with thyroiditis and thyroid antibodies Apparently the effect of thyroid pathology (thyrotoxicosis as well as adenomatosis) exceeds the effect of sex and age on the occurrence of thyroiditis and thyroid antibodies

In the present material correlation between the occurrence of thyroid antibodies and the existence of focal or diffus thyroiditis was found to be highly significant Specification of the antibody findings revealed a good correlation between thyroiditis and high and medium high titres of thyroglobulin antibody high as well as low titres of

microsomal antibody and the occurrence of more than one antibody On the other hand the isolated occurrence of low titres of thyroglobulin antibody and the isolated presence of the CA-2 antibody could not be correlated to the existence of thyroiditis

Direct correlation between the occurrence of thyroid antibodies and the existence of thyroiditis in autopsies has been described by *Weaver et al* (1966) and *Bastenie et al* (1967) who examined sera from their materials for thyroglobulin antibody alone and by *Goudie et al* (1959) who demonstrated the microsomal antibody alone Similar findings in surgical cases have been described by *Buchanan et al* (1961) and *Irvine et al* (1962) *Schade et al* (1960) found a correlation between thyroiditis and the thyroglobulin antibody but no correlation between thyroiditis and the microsomal antibody which however did not agree with the findings reported by *Buchanan et al* (1961) and by *Irvine et al* (1962) In the present material correlation between the existence of thyroiditis and the occurrence of the microsomal antibody was found to be good even if the latter was present in low concentrations in which this antibody could be demonstrated by the immunofluorescence technique but not by the complement fixation reaction

Several observations suggest that focal and diffuse thyroiditis are fundamentally identical diseases with only quantitative differences Firstly focal thyroiditis appears morphologically to be a patchy variety of the diffuse form secondly the same thyroid antibodies occur in sera from patients with focal and diffuse thyroiditis and thirdly physiological similarities between diffuse and focal thyroiditis may be demonstrated As previously mentioned myxoedema is a common occurrence in diffuse chronic thyroiditis and diffuse thyroiditis is probably the most common cause of myxoedema Clinically euthyroid patients with focal thyroiditis may display physiological signs of functional thyroid failure suggesting latent or preclinical myxoedema (*Bastenie et al* 1967) On the other hand only a minority of focal cases progress to become diffuse since the incidence of Hashimoto s disease and manifest myxoedema is much lower than might be expected if focal thyroiditis invariably progressed to envolve thyroid gland in its entenity This point has also been emphasized by *Williams & Doniach* (1961) who found that even though the overall incidence of focal thyroiditis increases considerably with advancing age the incidence of extensive cases hardly rises in the older age groups Moreover by serial thyroid biopsies from a number of thyroiditis patients *Mc Kory & Hamlin* (1961) found moderate progression of the lesions in only a few of their cases whereas no progression could be demonstrated in the majority of the patients

The presence of circulating antibodies (apart from the isolated occurrence of low titres thyroglobulin antibody and of CA 2 antibody) is a reflection of the existence of thyroiditis and high titres are suggestive of extensive thyroiditis On the other hand the mere presence of thy

roid antibodies is of limited clinical importance because most cases of thyroiditis are focal and asymptomatic and because the focal lesions show little tendency of progression into a disease of clinical importance, i.e. myxoedema or goitre.

SUMMARY

Focal but rarely diffuse chronic thyroiditis was found in 15 per cent of 477 consecutive autopsies of patients in whom clinical signs of thyroid disease were absent. The incidence was higher in women than in men and higher with advancing age. One or more of three thyroid antibodies (thyroglobulin antibody, microsomal antibody and CA-2 antibody) was found in 18 per cent of 1162 subjects without clinical signs of thyroid disease, the incidence being higher in women than in men and increasing with advancing age. The incidence of thyroiditis and thyroid antibodies did not vary with sex and age in thyroidectomized patients with thyrotoxicosis and atoxic goitre. In a material comprising 562 autopsy cases and thyroidectomized patients from whom sera as well as thyroid tissue were available, a presence of thyroid antibodies was found to be significantly more frequent in patients with focal or diffuse thyroiditis than in patients without thyroiditis. Even in low titres, as well as high and medium high titres of the thyroglobulin antibody, the occurrence of the microsomal antibody correlated well with the existence of thyroiditis, whereas the isolated occurrence of low titres of thyroglobulin antibody and of CA-2 antibody did not correlate well with the existence of thyroiditis. A correlation between extensive thyroiditis and high titres of thyroglobulin antibody and microsomal antibody was found.

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LUNG AMYLOIDOSIS INDUCED IN MICE BY TRANSPLANTATION OF CASEIN SENSITIZED SPLEEN CELLS

By

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During the development of amyloidosis in mice induced by casein treatment a certain sequence in the involvement of different tissues is the rule *Christensen & Rask Nielsen* (1962) followed the events in C3H mice treated with subcutaneous casein injections for up to 100 days by sequential killings and recorded the extent of amyloidosis histologically. The first trace of amyloid was found in the spleen after two to three weeks of casein treatment About a week later amyloid could be detected in the liver and after another week in the kidneys Later amyloid occurred in the adrenals intestine ovaries testes stomach pancreas and in the lymph nodes with the noticeable exception of the regional ones

In certain strains of mice old individuals develop amyloidosis spontaneously *Dunn* (1944) *Thung* (1957) and *West & Murphy* (1965) The topographic distribution of amyloid in such states is quite different from that of experimentally induced amyloidosis In West & Murphy's material of aged A/Sn mice lung amyloidosis marked the onset lesions in the tongue gastrointestinal submucosa lymph nodes and kidneys being frequent and early findings whereas spleen amyloidosis was occasional and late The authors cited above have pointed to the resemblance between senile mouse amyloidosis and primary amyloidosis in man

In a series of recent experiments we have demonstrated an amyloidogenic effect of the transplantation of spleen cells from casein sensitized mice to syngeneic recipients (*Werdelin & Ranløv* 1966 *Ranløv & Werdelin* 1967 *Werdelin & Ranløv* 1968 *Werdelin* 1968) In mice inoculated via the intravenous route with spleen cell suspensions from mice with spleen amyloidosis the occurrence of amyloid in the lung vessels was described It was concluded that these lesions were due to the transfer of preformed donor amyloid as they could be demonstrated in the lungs of mice dying within 10 minutes from the cell transfer

However small lumps of amyloid were also found in the lungs of mice inoculated with spleen cells from casein treated mice without amyloidosis These recipients had been treated with casein for 7 days + nitrogen mustard for 5 days subsequently to the cell transfer (*Werdelin* 1968) The purpose of the present experiments was to study 1) the morphology of such lesions 2) the time of their development after the cell transfer 3) the effect of inocula with unusually large numbers of cell aggregations on the development of lung lesions and 4) the state of the lungs in mice with severe and widespread amyloidosis induced by conventional methods

MATERIAL AND METHODS

Young C3H mice 5–15 weeks of age and of both sexes all belonging to the same inbred strain were used At the start of each separate experiment described below all mice involved were selected at random from a large number of cages and put into new cages 4–8 together males and females separately Throughout the experiment all mice were kept on a diet of oatmeal and tap water *ad libitum*

Four separate experiments were carried out 1) 14 mice each received 50 × 10⁶ spleen cells intravenously from donors treated with 7 casein injections The post transfer treatment was daily casein injections for 7 days and nitrogen mustard for 5 days All recipients were killed 12 days after the cell transfer 2) 28 mice each received 100 × 10⁶ spleen cells from a donor group treated with 7 casein injections the post transfer treatment was the same as that described above On each of the 12 days following the cell transfer 2 or 3 mice selected at random were sacrificed These recipients are referred to as sacrificed consequetively 3) A cell suspension was prepared from the spleens of a donor group treated with 7 casein injections Half of the suspension was centrifuged at 3500 revolutions per minute the other half was centrifuged at 1500 revolutions per minute The fast spun suspension containing an unusual large number of cell aggregates was injected intravenously into 25 recipients The slowly spun suspension was injected intravenously into 13 recipients dose per mouse 50 × 10⁶ cells Both groups of mice were treated with casein for 7 days after the cell transfer and the mice were all sacrificed on the 8th day following it 4) 53 mice were treated with casein for 4 weeks After the casein treatment they were treated with nitrogen mustard for 5 days All mice were sacrificed 33 days after the beginning of the treatment This group is referred to as comparison group Below the methods used for the treatment of donors and recipients the preparation of the cell suspensions the sacrificed and autopsies and the treatment of the mice in the comparison group are described in detail

Donor mice were treated for 7 consequetive days with one daily subcutaneous injection of 0.5 ml of 5 per cent casein in 0.25 per cent NaOH prepared according to *Christensen* (1963) The donor mice were killed by cervical dislocation on the day after the last casein injection The spleens were removed immediately and pooled in Ringer solution From each spleen a piece was taken for microscopy serving as a control for the development of amyloidosis

Spleen cell suspension None of the donor spleens used for the transfer experiments contained amyloid The spleens are cut into pieces and put into a loose fitting glass homogenizer with a quantity of Ringer solution about 4 times their volume By gentle manipulation of the piston a crude suspension is made This is filtered through a fine meshed metal sieve (1600 holes per cm²) The cells are washed 3 times by centrifugation at 2000 revolutions per minute for 3 minutes and resuspended in fresh Ringer solution (In experiment no 3 outlined above one half of the suspension was not filtered and during the washing it was centrifuged at 3500 revolutions per minute The other half of the suspension was filtered and the washing was performed by centrifugations at 1500 revolutions per minute) The number of nucleated cells per ml of suspension is counted in a Turk counting chamber and adjusted to the desired number of cells per ml by the addition of Ringer solution The trypan blue viability of the final suspension was found to be 80–85 per cent in each transfer experiment

Recipient mice received the spleen cells in 0.30–0.33 ml of the suspension by injection into the lateral vein and within two hours after the transfer each received an injection of 0.5 ml of 5 per cent casein in 0.25 per cent NaOH via the subcutaneous route. The casein injections were continued for 7 days. 24 hours after the last casein injection the treatment with nitrogen mustard was started. 0.05 mg of nitrogen mustard (Erasol IDO®) in 0.5 ml of isotonic saline was injected subcutaneously. This was repeated 48 and 96 hours later.

Sacrifice and autopsy. In the group of recipients killed consequetively two mice dying immediately after the cell transfer were autopsied and are referred to as recipients number 1 and 2 in Table 2 overleaf. On each day following the cell transfer 2 or 3 mice were selected at random and killed by cervical dislocation in slight ether anaesthesia. The last 3 mice were killed 12 days after the cell transfer having received 7 days of casein treatment + 5 days of nitrogen mustard treatment. At autopsy the right lung, spleen, liver, kidney, adrenal, small intestine and occasionally a mesenteric lymph node, ovary or testis were taken out, fixed in neutral formalin and embedded in paraffin.

Comparison group. 53 mice received daily casein injections 6 days a week, 24 injections in all, and were finally treated with nitrogen mustard during a 5 day period they received 0.05 mg of nitrogen mustard every second day. Casein, nitrogen mustard and injection route were identical with those applying to the recipient mice.

Histological methods. Sections were stained with haematoxylin eosin, pyronin methyl green, alkaline Congo red and periodic acid Schiff stain. In the comparison group only alkaline Congo red and periodic acid Schiff stained sections were prepared.

RESULTS

The results with regard to amyloid development in the three experiments are tabulated in Tables 1, 2 and 3. The spleen amyloidosis is evaluated quantitatively in degrees 1 to 6 according to *Christensen & Hjort* (1959) and the liver amyloidosis in degrees 1 to 4 by a scale used in previous investigations (*Werdelin & Ranløv* 1968).

TABLE 1

Prevalence, Topography and Mean Degree in Spleen and Liver of Amyloidosis in Recipients of 50 × 10⁶ Spleen Cells from Casein sensitized Syngeneic Mice Treated with Casein for 7 Days and Nitrogen Mustard for 5 Days after the Cell Transfer

	Spleen	Liver	Kidney	Adrenal	Intestine	Lung
Number of recipients with amyloidosis	14/14 (100%)	14/14 (100%)	7/13 (55%)	6/6 (100%)	9/12 (75%)	14/14 (100%)
Mean degree of amyloidosis	4.89 (4–5.5)	2.43 (2–3)	–	–	–	–

Number with amyloidosis / total number of organs available for study

All members of the group of 14 recipients inoculated with 50 × 10⁶ casein sensitized spleen cells and subjected to the full 12 days post transfer treatment developed a heavy and widespread amyloidosis and all developed amyloidosis in the lungs (Table 1).

The events taking place in the recipients during the posttransfer period can be studied in the *group of recipients sacrificed consequetively* (Table 2). It is evident from the table that the extrapulmonary amyloidosis commences in the spleen 4 days after the cell transfer

TABLE 2

Prevalence Topography and Degree of Amyloidosis in Recipients of 100 × 10⁶ Spleen Cells from Casein Sensitized Syngeneic Mice at Various Intervals after the Cell Transfer

Recipient no	Number of days after transfer	Spleen	Liver	Kidney	Adrenal	Intestine	Lung
1	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0
3	1	0	0	0	0	0	0
4	1	0	0	0	0	0	0
5	2	0	0	0	0	0	0
6	2	0	0	0	0	0	0
7	3	0	0	0	0	0	0
8	3	0	0	0	–	0	0
9	4	3	0	0	0	0	0
10	4	2	2	0	0	0	0
11	5	0	0	0	0	0	0
12	5	3	1	0	0	0	0
13	6	3	1	–	–	0	0
14	6	3	1	0	0	0	0
15	7	3	1	0	0	0	0
16	7	4	2	+	+	+	+
17	8	4	1	+	0	+	0
18	8	4	2	0	+	+	0
19	9	4	1	+	–	+	+
20	9	5	3	+	+	+	+
21	10	[illegible]	2	0	+	+	+
22	10	4	2	0	0	0	+
23	11	5	2	+	+	+	0
24	11	5	3	+	+	+	0
25	11	4	2	0	–	+	+
26	12	5	[illegible]	+	0	0	0
27	12	4	2	0	0	+	0
28	12	4	3	–	–	+	+

The initial spleen amyloidosis of degree 2 or 3 increases to degree 4–5 on the last days of the experiment. The liver amyloidosis can be detected one or two days after the onset of the spleen amyloidosis. It begins at the periphery of the liver lobule in and around the outer coat of the small vessels. After another two days amyloid appears in the kidneys, adrenals and intestine. The morphology and histochemistry of this extrapulmonary amyloidosis is identical with that described in a previous communication on transfer amyloidosis (*Werdelin & Ranlov* 1968).

In the lungs lesions are seen in a number of mice killed after the 6th day following the cell transfer. Small lesions are found as small bodies in the alveolar walls staining PAS positive and giving green birefringence with Congo red under crossed polars. They are oval and lie with their long axis along the alveolar wall (Figs. 1 & 2). Many of them seem to occlude the alveolar capillary, sometimes expanding it (Fig. The size varies, most being 10–50 mμ in the largest dimension.

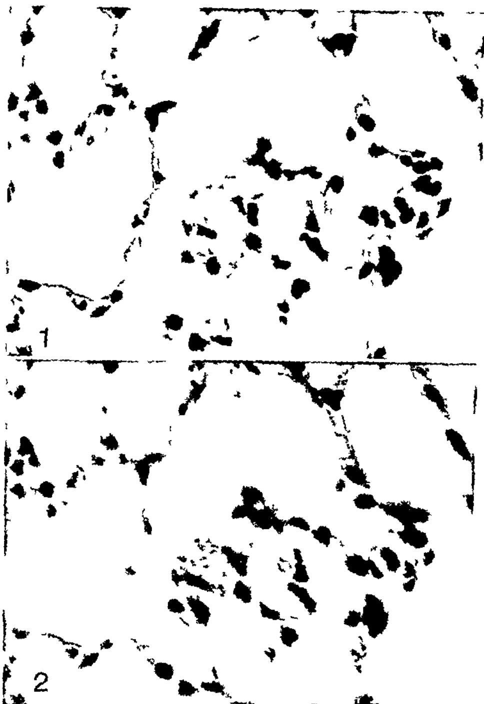


Fig 1 One large and three small bodies of amyloid in capillaries of alveolar wall Recipient sacrificed 10 days after the cell transfer Alkaline Congo red stain (× 840)

Fig 2 Same field as in Fig 1 Amyloid deposits having green birefringence in polarized light Alkaline Congo red stain (× 840)

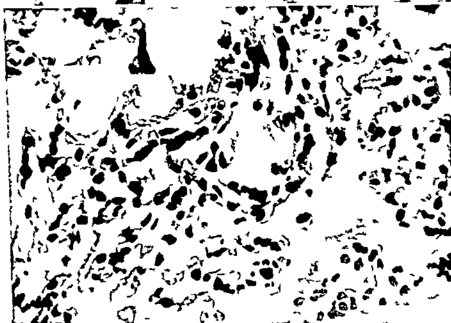


Fig 3 Amyloid deposit in alveolar capillary expanding vessel and compressing nuclei in the periphery Recipient sacrificed 10 days after the cell transfer Alkaline Congo red stain (× 840)

Fig 4 Large amyloid deposit in small arteriole in lung Recipient sacrificed 10 days after the cell transfer Alkaline Congo red stain (× 5 5[illegible]

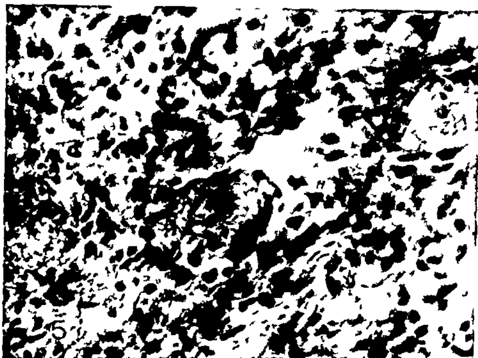


Fig 5

Three large deposits of amyloid in lung vessels. One (upper right) has a centre of Congo negative material and shows green birefringence in the periphery *only*. Frozen section from recipient of cell suspension rich in cell aggregations sacrificed *7 days after the cell transfer* (× 525)

amyloid material is homogenous but in the periphery of the deposits are found nuclei indistinguishable from those of the alveolar endothelium (Fig. 3). In the majority of recipients receiving the full post transfer treatment are found larger deposits of amyloid in the capillaries and arterioles occluding the vessels. Here too are found nuclei in the periphery of the amyloid (Fig. 4). The lung amyloid seems to be distributed evenly in the lung tissue and it shows no preference for peribronchial or subpleural regions. The number of deposits per field of vision varies.

TABLE 3

Prevalence, Topography and Mean Degree in Spleen and Liver of Amyloidosis in Intact Mice Treated with Casein for 5 Weeks and Nitrogen Mustard for 5 Days

	Spleen	*Liver*	*Kidney*	*Adrenal*	*Intestine*	*Lung*
Number of mice with *amyloidosis*	53/53 (100%)	53/53 (100%)	53/53 (100%)	39/39 (100%)	51/51 (100%)	15/53 ([illegible]7%)
Mean degree of amyloidosis	4.78 (4–5)	3.60 (2–4)		–	–	

Number with amyloidosis / total number of organs available for study

5

TABLE 4

Table over the Topography and Time of Onset of Spontaneous and Experimental Amyloidosis in Systematic Investigations of Mouse Amyloidosis

Authors	Strain of mice	Method of induction/age at spontaneous onset	First tissue to show amyloidosis/ length of induction period	Tissue in which amyloid is found in extensive stage	Involvement of lung
Dunn 1944	A hybrid ABC	Spontaneous 8 12 months	Around Brunner glands duodenum	Duodenum stomach colon intestine heart lung veins lung parenchyme ovaries testes adrenal endometrium skin kidney spleen liver	Yes in veins masses in the lobar periphery and in tumours
Thung 1957	O_{20} $(O_{20} \times DBA_f)$ F_1 DBA_f $(C_{57}BL \times DBA_f)$ F_1 $C_{57}BL$ DBA A	Spontaneous *6 12 months*	not stated	Kidneys adrenals ovaries testes spleen liver intestinal wall heart pancreas thyroid salivary glands vaginal wall fat skin	No
West & Murphy 1965	A/Sn	Spontaneous 4–5 months	alveolar septa	Lungs tongue stomach cervical lymph nodes colon mesenteric lymph nodes heart skin liver testes spleen bladder salivary gland	Yes *alveolar septa*
Deringer 1965	BL/Ly De	Spontaneous 15 months	not stated	Spleen duodenum ileum liver kidney ovaries uterus heart vaginal wall adrenal cortex lung tongue pancreas salivary glands bladder and testis Superficial cervical lymph node	Yes
Rask Nielsen Christensen & Clausen 1960	$(CBA \times DBA/2)$ F_1	Transplantation of reticulosarcoma	spleen 39 days	Spleen liver kidneys lymph nodes pancreas stomach intestine adrenals ovaries	No
Christensen & Rask Nielsen 1962	C3H	Casein injections	spleen 12–22 days	Spleen liver kidneys lymph nodes ovary adrenals pancreas stomach intestine	No

In mice dying immediately after the cell transfer numerous large emboli are found in the lungs consisting of smudgy necrotic material and fibrin These do not give the histochemical reactions of amyloid In mice surviving the cell transfer are found a few small arterioles and capillaries occluded by PAS positive material which does not give the staining reactions of amyloid

In the third experiment where *cell aggregations* were transferred the two suspensions were studied in the counting chamber before the inoculation It was found that the fast spun suspension contained an unusual large number of cell aggregations (1–3 per 16 small fields against 0–1 per 16 small fields in the slowly spun) As aggregations were arbitrarily reckoned groups of 20 or more agglutinated cells

None of the donor mice had developed amyloidosis and 9 recipients sacrificed within 48 hours after the cell transfer had not developed lung lesions On the 7th day following the cell transfer 4 recipients all inoculated with the fast spun suspension suddenly died showing neurologic disturbances and respiratory distress They were not autopsied All mice therefore were sacrificed on the 8th day after the cell transfer

The recipients in the two groups now numbering 12 and 13 mice respectively all developed amyloidosis to the same degree in the spleen (mean degree 3) liver (mean degree 1) kidneys adrenals and intestine In the lungs of the recipients of the fast spun suspension however large lumps of amyloid in the vessels were about twice as numerous as in recipient of the slowly spun suspension In some of the amyloid deposits was seen a central core of PAS positive material which did not stain with Congo red and which did not display green birefrigence in polarized light (Fig 5)

All mice in the *comparison group* developed a heavy and wide spread amyloidosis (Table 3) The spleen amyloidosis averages degree 5 and the liver amyloidosis degree 3–4 Renal suprarenal and intestinal amyloidosis is constant and more pronounced than that of the most affected recipient mice In the lungs of 15 mice were found a few small bodies of amyloid similar to those observed in the recipients lungs

The histochemical reactions of amyloid in the lungs and the extra pulmonary amyloid are the same It is PAS positive Congo positive and in Congo stained sections are found green birefringence in polarized light It is not metachromatic with methyl violet

DISCUSSION

The occurrence of amyloid in the lungs of mice with experimental amyloidosis is remarkable and does not seem to have been described previously It was found in recipients of casein sensitized cells as well as in members of the comparison group with amyloidosis induced

by conventional methods But whereas lung lesions were numerous and were encountered in 100 per cent of the recipients of cells they were found in only 28 per cent of the mice of the comparison group and when found were few and small That lung amyloidosis is not simply a feature of a heavy and widespread amyloidosis proportional to its degree is evident from the fact that the extrapulmonary amyloidosis in the comparison group was more intense than that of the recipients

In Table 4 is listed the results obtained by others with regard to the distribution of amyloidosis in mice It will be seen that *Dunn* (1944) and *West & Murphy* (1965) have observed amyloid in the lungs in systematic investigations of the spontaneous amyloidosis occurring in aged mice of certain strains while *Thung* (1957) investigating senile amyloidosis in a number of strains and hybrids between them did not find amyloid in the lungs In the spontaneous amyloidosis in A/Sn mice (*West & Murphy* 1965) the lung amyloidosis is essentially alveolar and described as being located in the alveolar septa between the septal cells and the endothelium The authors cited above remark upon the similarity with regard to topographic distribution between the spontaneous amyloidosis in old mice and primary amyloidosis in man *Heston & Deringer* (1948) finding different frequences of age amyloidosis in a number of mouse strains and hybrids between them draw the inference that this kind of amyloidosis is inherited and probably dependent upon a single recessive gene

The extensive amyloidosis induced in mice by prolonged casein treatment (*Christensen & Rask Nielsen* 1962) or by subcutaneous transplantation of reticulosarcoma (*Rask Nielsen & Christensen* 1960) is not associated with lung lesions it invariably starts in the spleen and its pattern corresponds to secondary amyloidosis in man In mice with severe and widespread amyloidosis resulting from treatment with casein followed by nitrogen mustard the lungs did not show amyloidosis (*Werdelin & Ranlov* 1966) Casein induced mouse amyloidosis accelerated with whole body X irradiation does not comprise lung amyloidosis (*Christensen & Hjort* 1959) It is possible though that investigators not using the very sensitive method of polarization microscopy of Congo stained sections may have overlooked small lumps of lung amyloid

The present finding of numerous small amyloid precipitates in the lungs of cell inoculated mice thus distinguishes this amyloidosis from other forms of experimentally induced amyloidosis although a few similar lesions could be detected in the present comparison group in 28 per cent of these mice with heavy and widespread amyloidosis induced by conventional methods An explanation of the development of such lung lesions may be sought in the experimental procedure of intravenous injection of spleen cells In a paper on amyloidosis after transfer of spleen cells *Werdelin & Ranlov* (1967) stressed the pa

rallelity between the topography of the amyloidosis and the distribution of the transferred cells Lymphokinetic studies of the fate of intravenously injected labelled lymphoid cells have clearly demonstrated the arrest in the lung capillaries of many of the injected cells immediately after the cell transfer (*Weisburger et al* 1951) (*Keohane & Metcalf* 1958) (*Diderholm* 1961) (*Ranlov & Werdelin* 1967) Later most of the cells trapped in the lungs are released and increasing numbers can be traced to the spleen and liver of the recipient Some of the transfused cells however are retained in the lungs *Shorter & Bollman* (1960) found radioactivity in the lungs as late as 120 hours after the transfer of P^{32} labelled lymphocytes in rats

The fact that larger lumps of amyloid in the lungs are found to be twice as numerous in recipients of a suspension rich in cell aggregates as in recipients of a suspension of the usual kind points to the number of cells retained in the lungs as being a determining factor for the development of the lesions

We suggest that the amyloid in the lungs is produced locally in close relation to retained spleen cells or their breakdown products A similar conclusion was drawn by *Hultgren et al* (1967) who also found an atypical distribution of the developing amyloid in transfer experiments involving the intraperitoneal inoculation of spleen cells from azo casein sensitized donors to recipients treated with azo casein Amyloidosis in spleen and liver was severe and deposits were found in the mesentery omentum and abdominal lymph nodes *i e* the organs situated along the major portal and lymphatic drainage pathways of the peritoneal cavity From their observations the authors conclude that the transfer amyloidosis results from an interplay between the sensitized donor cells and the RE cells of the recipient with the latter cells determining the final location of amyloid deposition

The mechanism by which the transferred cells promote amyloidosis in the recipient is not clearly understood The transferred cells need not be viable *Janigan & Druet* (1967) have demonstrated an amyloidosispromoting effect of the intraperitoneal transfer of disrupted azo casein sensitized spleen cells to lethally X irradiated C3H mice *Ranlov* (1967) testing different fractions of the disrupted spleen cells separately found the effect bound to the nuclear fraction *while the* cytoplasmatic fraction was not amyloidogenic The effect of the nuclear fraction prepared according to the method described by *Medawar* (1963) was found to be DNAse resistant

From this it is evident that the recipients own RE cells must play a role in the development of the transfer amyloidosis As transferred heat damaged cells and fragments of disrupted cells are probably eliminated by the recipient s phagocytes a closer study of these cells during the development of the lesions may prove fruitful

SUMMARY

The development of small bodies of amyloid in the lungs of mice inoculated with casein sensitized spleen cells is described The lung lesions are part of a widespread amyloidosis affecting the spleen liver kid neys adrenals and intestine They become visible shortly after the spleen and liver amyloidosis and give the typical staining reactions of amyloid When the inoculum contains many aggregations of cells the lung lesions are numerous It is concluded that the lung amyloid is probably manufactured in close relation to transferred cells trapped in the lung vessels after the intravenous inoculation

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FOETAL GENETIC DIAGNOSIS DEVELOPMENT OF TECHNIQUES FOR EARLY SAMPLING OF FOETAL CELLS

By

JAN MOHR

Received 11 x 67

The advances of recent years which have made possible the diagnosis in cell culture of sex chromosomal aberration and certain biochemical defects and traits represent important new possibilities in counter acting genetic disease If cells of foetal origin may be procured safely from a sufficiently early stage of development and if legislation per mits therapeutic abortion in case of eugenic risk pregnancy may be interrupted when the foetus is found to be genetically defective

In the first place this is of interest in certain relatively rare cases of serious initial risk such as in kindreds with the type of translocation carrier which implies a high probability of Down s syndrome in the offspring and in families in which both parents are carriers of one of those recessive biochemical disorders that may be diagnosed in cell culture

The procedure might however come to develop into a routine as regular as for instance the use of Wassermann s reaction if safe and sufficiently convenient techniques for early foetal genetic diagnosis could be developed

In the special case of sex linked disease a kind of foetal diagnosis has been accomplished already about a decade ago (*Fuchs & Riis* 1956) not many years after the discovery of sex chromatin by *Barr & Bertram* (1949) In carriers for haemophilia the amniotic fluid was sampled by suprapubic puncture and the cells in this fluid which are of foetal origin examined for sex chromatin Therapeutic abortion was given in case of male sex the risk of haemophilia being fifty per cent for this

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Thanks are du to Dr *Valdemar Madsen* M D head of the Department of Obste trics Gentofte Amtssygehus and Dr *Rolf Plesner* of the same Department for helpful discussions and for providing material of foetal ti ues D C *Long* High land View Hospital has given valuable advice concerning the fibre optics as has Dr *D A Pontarelli* of the IIT Chicago who also c nt but d m te ial for initial experiments concerning the fibre optics

sex In cases of female sex for which the risk of haemophilia is practically nil the pregnancy was allowed to go to term

The suprapubic puncture is only practicable at a rather advanced stage of pregnancy less favourable with regard to therapeutic abortion than earlier stages and therefore scarcely applicable as a routine procedure Another limitation is that the simple puncture technique only provides cells of the amniotic fluid While sex chromatin is readily recognizable in these and the sex of the foetus therefore diagnozable it appears that even the best results regarding cell culture from amniotic fluid ar not fully satisfactory (*Steel, Breg & Roy* 1966) Therefore techniques are needed for procurement of foetal cells other than those of the amniotic fluid

For better visualization of the technical problems in point it would appear reasonable to give a few facts about the membranes concerned up to three month stage

An egg of five weeks including foetal membranes and embryo proper may be sketched as a fluid filled deciduachorion sac of size as a small walnut containing a hazelnut sized amniotic sac encasing a bean sized embryo proper The wall of the decidua chorion sac is composed of an outer layer of maternal origin and facing the uterine cavity the decidua capsularis and an inner layer of foetal origin the chorion this latter layer is at this early stage of pregnancy richly supplied with chorionic villi that protrude into the decidua capsularis At this stage the amnion could scarcely be reached by any practicable biopsy procedure since it is deep within the egg

It might however be possible already at this stage to obtain for culture a small piece of the chorion decidua tissue that constitutes the outer wall of the egg and faces the still open uterine cavity provided an instrument permitting accuracy in operation and efficient closing of the lesion were available

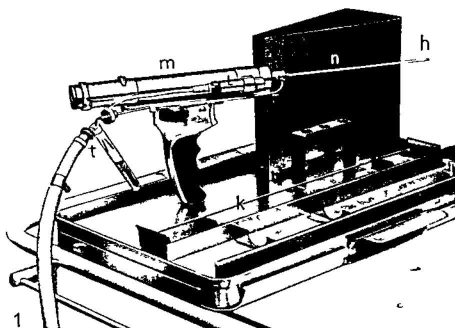
The maternally derived decidua cells might constitute a complication depending upon whether culturing conditions would favour the cells of foetal origin or not The situation in this regard would not be difficult to evaluate since the required full preponderance of foetal cells would imply an approximate 1 1 sex ratio as measured by the occurrence of Y chromosomes in the cultures

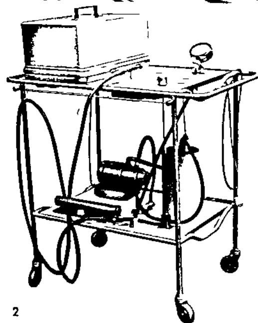
The amniotic sac grows rapidly A month later at the end of the second month of pregnancy it fully fills out the volume of the decidua

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Fig 1

Upper field Biopsy instrument proper Tubular needle (n) hole (h) through which adjacent tissue is inspected and biopsy performed low power microscope (m) tubular knife for biopsy (k) tube (t) for colection of amniotic fluid *Lower left field* Instrument enclosed in steel casing mounted together with auxiliary equipment *Lower right field* Field of vision and power of resolution of the fiber optics as demonstrated by a sample of human hair as the object


h
m
n
t
k
1


2



chorion sac At this stage a biopsy of the egg wall could contain three categories of cells Maternally derived cells of the decidua capsularis and foetal cells from the chorion and from the amnion

At the end of the third month of pregnancy the surface of the egg *i e* the maternally derived decidua capsularis comes into full contact with the walls of the uterine cavity and there is a fusion of decidua capsularis with the decidua parietalis which lines the uterine cavity so that from this stage on the uterine cavity is closed thus only the part of the egg directly above the cervical canal is now accessible through a natural opening or cavity

While the decidua parietalis is now firmly fused with the decidua capsularis, as is this latter layer with the chorion the amniotic membrane is rather loosely attached to the chorion from which it is separable even by a slight mechanical exertion possibly this may offer a means of obtaining a sample of amniotic membrane free of any admixture of maternal cells

We have for some time been engaged in a project to develop techniques for sampling foetal cells not later than the third month of pregnancy aiming in particular at a sample from foetal membranes

While suprapubic puncture at a more advanced stage of pregnancy would be the technique of choice only an approach per vaginam would appear practicable in earlier stages of development It remains to be decided whether the best approach is through the cervical canal or by way of fornix anterior or another part of the recess around the portio vaginalis uteri and through the muscular wall of the uterus

It was thought that puncture and biopsy of foetal membranes through the cervical canal could imply a risk of leakage of the amniotic fluid or other complications such as infection with a corresponding risk of unintentional provocation of abortion

If such difficulties could be surmounted which would probably require an instrument equipped with a special closing device for the defect left by the biopsy procedure an approach through the cervical canal would appear preferable Very early stages of pregnancy could then possibly be made available for foetal genetic diagnosis although early stages might imply special difficulties such as admixture of maternal cell in the biopsy

It was however decided to consider first an approach through the muscular wall of the uterus partly because this would be closest to the cited already practised suprapubic puncture which is known to be compatible with a further normal development of the foetus

An instrument developed for this purpose is shown together with auxiliary devices in the figure It was developed with the assistance of Ole Dich instrument maker Kirke Hyllinge who has contributed outstandingly as regards the mechanics of the instrument optical parts have been made by Josef Lauber Rüsselsheim

The instrument comprises a tubular thin walled needle (n) of 2 1

mm outer diameter and a length of 202 mm from tip to holder by means of which puncture would be made per vaginam

The needle is supplied with a hole (h) near the tip from which a picture of the tissue facing the hole is transmitted to a low power microscope (m) through a coherent bundle of 10 micron glass fibres encased in a second thin walled tube of 1 7 mm outer diameter

Illumination is supplied by a lamp (1) from which light is trans mitted through a prism arrangement into a separate set of glass fibres encased in the same tube as the picture transmitting coherent bundle

The optical part of the instrument is removable in toto while the needle remains in the position attained through the inspection per mitted by the optic device The lower right part of the picture demon strates the size of the field of vision and the resolution which is limited in accordance with the diameter of the glass fibers the chosen object being a sample of human hair

A tubular knife (k) may now be introduced in place of the optic device to cut off and collect any foetal membrane tissue bulging into the hole near the needle tip Suction is applied throughout this proce dure to make the tissue bulge into the needle A sample of amniotic fluid may be procured by suction into a glass tube (t) fitted to the instrument

The full arrangement is shown in the lower left part of the picture including various auxiliary equipment The instrument proper is en cased in a steel container for easy conveyance to and from sterilization

The instrument has so far been tested in various model experiments It is now to be tested in a series of pregnancies in which therapeutic abortion has been decided upon in advance to decide whether it is applicable or not in its present shape

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INFLUENZA VIRUS HOST ANTIGEN IN CHICKEN TISSUES

By

GUNNAR HAUKENES

Received 8 ix 67

The host antigen of the haemagglutinin of influenza virus grown in the chicken allantoic cavity is present in non infected allantoic fluid of 12 day old chicken embryos at a concentration of about 10 μg per ml (1 7 8) Lower values have been found for the amniotic fluid (2) while the chicken bile contains as much as 1 mg of host antigen per ml (3) Liver extracts and stomach contents were also found to contain this antigen

In an attempt to find the origin of the host antigen tissues from chicken embryos of different ages have been examined Host antigen from bile and amniotic fluid has been purified and compared chemically with allantoic host antigen

MATERIALS AND METHODS

Tissue extracts Chicken embryo tissue was ground in a mortar and thereafter extracted with saline buffered at pH 7 2 with 0 01 M phosphate (PBS) for 30 mins at room temperature 100 mg wet weight of liver and whole embryo and 10 mg of mesonephros and kidney were extracted with 1 ml PBS The extracts and the yolk sample were thereafter treated with 0 25 per cent trypsin (Difco 1 250) at pH 8 1 overnight and heated at 100 C for 10 mins Haemagglutination inhibitors were thus prevented from interfering in the serological tests

Haemagglutination inhibition blocking (HIB) test This was used to assay the host antigen and performed as described in (2 1?)

Double diffusion in agar was performed according to *Ouchterlony* (10) The antiserum was applied 1–2 hours before the antigen samples as one line is produced by the slowly diffusing γM globulins (5)

Antisera Two rabbit immune sera were used one against the purified host antigen and the other against influenza A/PR–8 virus infected allantoic fluid Both sera produced two precipitation lines with the host antigen as described earlier (5) as well as strong haemagglutination inhibition against B/Lee virus

Purification of host antigen This was performed along the same lines as described earlier (6) Some special problems involved in purification of host antigen from amniotic fluid and bile are mentioned below

Chemical analyses Neutral sugar hexosamine fucose uronic acid ester sulphate and proteins were estimated by the methods referred to earlier and the same paper chromatography procedures were used (7) *Fatty acid esters* were determined as described in (11)

EXPERIMENTS AND RESULTS

Chicken Embryo Tissues and Fluids

Extracts were prepared from the following tissues: Whole embryo, liver, mesonephros and kidney. In addition samples were taken from amniotic fluid, allantoic fluid, bile, stomach contents and yolk. The collected tissue extracts and fluid samples were stored frozen until all samples could be examined simultaneously.

Before being examined by the HIB test the samples were checked for unspecific inhibitors and agglutinins. Strong inhibitors were found in the amniotic fluid and the bile. In the amniotic fluid the inhibitors appeared in 12–13 day embryos, at the time when albumen enters the amniotic sac. However, at that time HIB active material could be demonstrated in higher dilutions than the haemagglutination inhibition and special treatment to destroy inhibitors was not necessary. Haemagglutinins were found in bile and yolk material. In bile the HIB titres were higher than the haemagglutination titres. Yolk material caused haemagglutination of vaccinia negative fowl red cells to a titre of 1/32 to 1/128, an activity which could not be removed by trypsin treatment or absorption with red cells. Therefore the HIB titration had to start from 1/32 to 1/128 with these samples.

HIB titration and agar precipitation patterns for tissue extracts and fluids showing HIB activity are shown in Table 1. The amounts of host antigen can be calculated from the HIB titres according to a titre of 1:32 000 given by 1 mg per ml of purified host antigen. The same solution will produce precipitation line(s) by double diffusion up to a 1:500 dilution.

It appears from Table 1 that the host antigen is present in the allantoic fluid from the first day of examination, *i.e.* the 6th day. In the amniotic fluid, however, no host antigen could be demonstrated until the 11th day of incubation. Both fluids showed rising titres up to almost similar values on the last day before hatching. The rising titres may partly be explained by a concentration of the material due to water absorption.

When the precipitinogen content of the two fluids is compared the difference is less pronounced. It is seen that precipitation lines were obtained with an amniotic fluid sample of relatively low HIB activity (1/32).

No connection exists between the allantois and amnion. However, to test for a possible uptake of substance from the allantois and excretion in the amnion, polysaccharide A of *Staphylococcus aureus* was injected into the allantoic cavity. This polysaccharide is, like the host antigen, a strongly acidic polysaccharide being a teichoic acid. Polysaccharide A could be demonstrated by ring test precipitation in the allantoic fluid for one week, but no antigen material appeared in the amniotic fluid.

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The 6 and 7 day embryos were examined as a whole as the organs were too small to give sufficient amounts for quantitative testing Both extracts contained host antigen

It was not possible to get samples of bile before the 11th day There fore a combined liver/gall bladder extracts was examined from 8 9 and 10 day old embryos Host antigen was demonstrated in two of these extracts Liver extracts showed varying titres in most samples the HIB activity was low and only one extract gave precipitation in agar

Owing to shortage of material no attempt was made to quantitate the host antigen in the bile The contents of three gall bladders were taken up in 1 ml of saline The rising HIB titres seen in Table 1 most probably reflect only an increasing amount of bile

Most mesonephrons extracts contained no host antigen and when found the HIB titres were so low that contamination cannot be ex cluded Both kidney extracts however contained small amounts of host antigen

Very high titres of host antigen were obtained with yolk material from the 17th day The appearance of the substance in the yolk coin cided with a greenish discoloration of the same At this step of develop ment the yolk sac opens into the gut to be emptied there A regurgi tation of bile into the yolk sac is the reasonable explanation of the pre sence of the host antigen

All other tissues failed to produce HIB activity or precipitation lines in agar

Purification of Host Antigen from Amniotic Fluid and Bile

Amniotic fluid was collected from 13–14 day chicken embryos The allantoic fluid was removed and the chorioallantoic cavity was tho roughly washed with saline before withdrawal of the amniotic fluid In pilot experiments it appeared that the amniotic fluid at this embryonic stage had a very high content of protein Freshly collected amniotic fluid showed an HIB titre about 4 times higher than frozen samples and the activity could not be regained by heating to 37 C The host antigen appeared to be partly inactivated by proteins as pronase treatment of frozen samples caused a 2 to 4 fold increase in HIB titre Earlier experiments on purification of amniotic host antigen pointed in the same direction When proteins were removed by ion exchange chromatography in these experiments an unexpected increase in the amount of serologically active material was obtained

Precipitation of the host antigen at pH 4 was not very successful as 50 per cent of the serologically active material remained in the super natant and a prodigious amount of precipitate was obtained There fore both the precipitate and the supernatant had to be subjected to further purification on DEAE cellulose and DEAE-Sephadex columns Alcohol precipitation was omitted since preliminary experiments showed that is was very difficult to dissolve the host antigen from the

precipitate Repeated chromatographic runs on DEAE cellulose and DEAE Sephadex were required After lyophilization the purified material was a white powder with a slight greenish discoloration

The chemical composition of the resulting semi purified host antigen is shown in Table 2 and compared with the data obtained with purified allantoic host antigen in earlier experiments (7)

TABLE 2

Chemical Composition of Amniotic and Allantoic Host Antigen

	Mg pr 100 mg dry weight						
	Protein (Folin)	Galactose	Uronic acid	Fucose	Hexosamine (as N acetyl)	SO_4	HIB titre
Allantoic host antigen	58	270	0	35	350	120	1/32 000
Amniotic host antigen	*56*	*200*	*28*	*5*	*198*	*100*	*1/4 000*

It is seen that we were not able to get rid of all uronic acid from the amniotic host antigen The ion exchange chromatography clearly indicated that the compound containing uronic acid was an impurity as the peak of HIB activity was eluted at a lower NaCl molarity than the peak of uronic acid Complete separation was not possible by this method without losing too much of the serologically active material The most striking difference compared with the allantoic host antigen is the low fucose content and the correspondingly low HIB titre A parallel reduction of the precipitating activity on double diffusion in agar was also obtained

Paper chromatography of acid hydrolysates showed the same monosaccharide composition as found for allantoic host antigen namely galactose glucosamine galactosamine and fucose Visually estimated galactose glucosamine and galactosamine gave spots of the same strength as in hydrolysates of allantoic host antigen The fucose spots however were very weak

Less than 60 per cent of the material could be accounted for by sugars SO_4 and peptide material The material obviously contained some bile pigment causing the greenish discoloration Some lipid contaminations were also demonstrated 18 per cent calculated as tripalmitin

Even greater difficulties were met with on purification of the host antigen from the bile The host antigen could be precipitated from the bile with acid at pH 4 as well as with alcohol It was however very difficult to dissolve the antigen from the precipitates Considerable amounts of active material were therefore lost during purification which otherwise followed the same lines as described above for the

amniotic host antigen The resulting semi purified substance which still contained some uronic acid and protein was examined chemically and was found to be a sulphated mucopolysaccharide with the same monosaccharide composition as found for the allantoic host antigen

DISCUSSION

The host antigen was present in the allantoic fluid apparently from its first production *i e* before the 6th day Bile is not excreted into the gut till the 15th day of incubation (9) Our embryos were also examined for bile in the gut The whole gut was not discoloured by bile pigment until the 16th to 18th day The host antigen of the early allantoic fluid can therefore not originate from bile excretion

Most likely the host antigen is excreted into the allantoic cavity by the mesonephros We were able to demonstrate only trace amounts of host antigen in extracts of the mesonephros This does not exclude an excretion as only minute amounts of urine might be present in the small narrow tubuli of the mesonephros Later host antigen seems to be excreted by the kidney

Although high contents of host antigen were demonstrated in the bile only trace amounts were found in liver extracts both in the present experiment and in other experiments not cited above The reason for this is not clear One might speculate that the host antigen is excreted from the liver as a neutral polysaccharide which becomes sulphated in the gall bladder On the other hand it is not likely that the sulphate groups are essential for the serological reactivity of the antigen

The presence of the host antigen in the whole embryo must be ascribed to its presence in the bile its presence in the yolk has already been commented on

More obscure is the presence of the host antigen in the amnion as there is no communication between amnion and allantois No explanation of this can as yet be forwarded

Host antigen in allantois amnion and bile showed the same main sugar and sulphate composition The amniotic host antigen showed very low fucose value and a correspondingly lower HIB activity This points to fucose as the antigenic determinant group Recent experiments with enzymes have confirmed this (4)

The reason for the low fucose content in the purified amniotic host antigen is not clear The fucose linkage is rather labile and some fucose might have been split off in the course of the prolonged purification procedures Another possibility is an enzymatic activity of the amniotic fluid A host antigen splitting enzyme is present in the intestinal fluid (3) and may have been brought up into the amniotic fluid by the embryo

Regarding the origin and biological importance of the host antigen

in the chicken organism we can at present only conclude that it seems to be an excretion product Most probably a precursor substance is altered chemically in some way before or during the excretion and thereby changed antigenically

SUMMARY

The host antigen of influenza virus was demonstrated in the allantoic and amniotic fluids the bile and the yolk of chicken embryos The host antigen is present in the allantois before any bile is excreted into the gut The host antigen found in the yolk arises from regurgitation of bile into the yolk sac Very small amounts of host antigen were also demonstrated in liver mesonephros and kidney tissues The host antigen has thus been demonstrated only in excretions or excretion organs

The chemical composition of the host antigen in bile and amnion was the same as that of the allantoic host antigen aside from a lower fucose content and HIB activity of the amniotic antigen

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RESISTANCE OF TOXOPLASMA GONDII ENCYSTED IN PORK

By

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Raw meat is generally accepted as a possible source of human toxoplasmosis. Before consumption, however, meat is usually submitted to various preparatory methods, i. e. frying, boiling, smoking or salting. In order to examine the resistance of encysted toxoplasma to such ordinary methods of preparation, the present studies were performed in cooperation with The Danish Meat Research Institute, Roskilde, Denmark.

Toxoplasma is not very resistant to physical influence and chemical agents.

Heating: *Eichenwald* (1948) examined proliferative toxoplasma in milk and found that no viable parasites remained after 15 minutes at 50° C or 10 minutes at 54° C. *Manwell & Drobeck* (1952) found that proliferative toxoplasma in mouse peritoneal exudate survived 50° C for five minutes, but not 55° C. *Kass* (1954) found mouse peritoneal exudate to remain infective after 5 minutes at 50° C, but not after 10 minutes. *Rawal* (1959) reported that cysts were killed in 10 minutes at a temperature of 60° C. *Jacobs et al.* (1960) examined cysts in a brain suspension from rats chronically infected with the Beverley or RH strain and found no infectivity to mice after one hour at 50° C.

Freezing of toxoplasma has been studied by many authors, mainly because maintenance of strains by storage at —70° C would be easier and safer than by animal passages. *Manwell & Edgett* (1947) found that toxoplasma survived freezing to —70° C and successive thawing, and *Weinman & Mac Allister* (1947) reported that toxoplasma would survive this procedure even when it was repeated six times in succession. However, storage over a longer period was not possible until *Weinman & Chandler* (1954) succeeded in storing toxoplasma at —70° C for at least 60 days. In none of these studies is it stated clearly whether the proliferative or the cystic form of the parasite was used. *Eyles et al.* (1956) worked with proliferative toxoplasma in mouse peritoneal exudate and demonstrated that it was possible to store the parasite for up to 209 days after freezing to —70° C in 5–10 per cent glycerol. *Jacobs et al.* (1960) examined encysted toxoplasma in a brain suspension containing Beverley and RH cysts and demonstrated that freezing to —15° C and thawing after 24 hours caused a complete loss of infectivity.

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Storage at refrigerator temperature is of importance not only in laboratory procedures but also epidemiologically *Levaditi et al* (1929) stored brains from rabbits that died in the acute stage of toxoplasmic encephalitis and found that after 48 hours they were no longer infective *Sabin & Olitsky* (1937) kept mouse brains in Tyrodes solution in the refrigerator for 14 days and found that they were still infective when used for inoculation The strain had originally been isolated from the brain of a guinea pig *Manwell et al* (1945) found that toxoplasma in dead animals would survive 24 hours in the refrigerator but seldom 48 hours It is not stated whether these animals had died from acute toxoplasmosis or had been chronically infected *Manwell & Drobeck* (1952) used sterile skim milk for storage of infective mouse brains and found no loss of infectivity after 18 days in the refrigerator Furthermore the infections produced in mice by material stored in this manner were as heavy and developed as rapidly as infections in mice inoculated with fresh material *Kass* (1954) found carcasses of animals that died from acute toxoplasmosis to be infective after 6 days at +4 C and *Jacobs et al* (1960) found survival of cysts in macerated brains after 68 days at +4 C

In this laboratory mouse peritoneal exudate is routinely stored at +4 C and after three weeks it still contains viable parasites

Transportation of material infected with toxoplasma may give information concerning the resistance of the parasites *Stim* (1961) reported that proliferative toxoplasma in a mouse peritoneal exudate packed in ice was shipped by air from Copenhagen to Australia The material was still infective after 7 days Toxoplasma in a human lymph node was still infective after two days at room temperature

Various suspending media have been examined *Jacobs et al* (1952) suspended proliferative toxoplasma of the RH strain in saline and noted a loss in infectivity after 2–3 hours at +5 C whereas a suspension in saline containing 10 per cent serum or 1 per cent neopeptone broth was infective after 24 hours at room temperature *De Roever Bonnet* (1960) found milk and 10 per cent human serum in saline to be of equal value for storage of proliferative toxoplasma with only a slight decrease of infectivity after 24 hours at +4 C

Osmotic pressure changes are tolerated only within narrow limits *Kass* (1954) demonstrated that proliferative toxoplasma would still be infective after 30 minutes at 37 C at NaCl concentrations from 0 2 to 4–5 per cent *In distilled water* there was no infectivity after 30 minutes at 37 C *Jacobs et al* (1960) placed toxoplasm cysts in distilled water and found that no infectivity remained after 30 minute At NaCl concentrations from 0 1–2 1 per cent isolated cysts lost their infectivit after 24 hours at room temperature *Stone & Manwell* (1963) examined the viability of proliferative toxoplasma in distilled water and found that even when present in large numbers they did not survive longer than 10 minutes

Drying *Manwell et al* (1945) dried proliferative toxoplasma over I O at room temperature and did not find any infectivity after 24 hours *Van Thiel* (1949) found that a toxoplasma positive brain *smeared on a slide in a thin layer and desiccated* for two days still contained virulent parasites It is not clearly stated whether these were proliferative or encysted *Kass* (1954) examined proliferative toxoplasma in mouse peritoneal exudate and after drying on a slide at room temperature there were no viable parasites after two hours and a half *Jacobs et al* (1960) allowed brains of mice with numerous toxoplasma cyst (Beverley strain) to dry in dishes at room temperature After one day at 18 20 C the brain were no longer infective

Resistance to *alcohol* and *phenol* was examined by *Manwell et al* (1945) The *found that 70 per cent ethyl alcohol or 5 per cent phenol* would kill proliferativ toxoplasma in 10 minutes

Resistance of toxoplasma to gastric juice is of considerable epidemiological interest *Weinman & Chandler* (1956) exposed toxoplasma in liver to artificial gastr juice and found viable organisms after 4 hours It is not stated *whether* the parasites were proliferative or encysted *Wolfführ* (1956) studied the effect of n mal gastric juice on proliferative toxoplasma and found that there was a compl t loss of infectivity after 30 minute *Jacobs et al* (1960) reported that prolifer ti parasites were killed after one hour in a pepsin *HCl* *lution but* survived at lea t *three hours in trypsin* Encysted toxoplasma survived two and sometimes thre hours in pepsin and up to six hours in trypsin indicating that they might al survive a normal human gastric passage

Of equal epidemiological interest i the *resistance of encysted t x plasma* *ii*

meat *Garnham & Lainson* (1960) heated a whole leg from an artificially infected sheep in an electric oven for 40 minutes at low temperature Although part of the meat was completely uncooked isolation of parasites was not possible Unfortunately no information is given about the temperature of the oven or of the meat nor is it stated for how long the meat was heated *Sommer et al* (1965) stored meat at +4 C and found viable parasites after 3 weeks and toxoplasma was found to survive 3 days at −15 C No parasites could be isolated from professionally smoked or salted meat or meat products Heating was studied on cubes of meat about 5 × 5 × 5 cm heated in oil at 160–170 C. Encysted toxoplasma was found to survive 5 minutes but not 7 minutes No information is given about the maximum temperature obtained in the centre of the meat cubes Furthermore it should be noted that some of the meat samples used in these experiments were injected artificially with a brain suspension containing encysted toxoplasma thus making a comparison with natural conditions difficult

MATERIALS AND METHODS

Selection of a Naturally Infected Swine

In order to imitate normal conditions meat from a naturally infected swine was used

In former serological and isolation studies it was found that meat from a pig with a titre of 1 250 in the dye test would provide a reasonable possibility (about 80 per cent) of isolating toxoplasma (*Work* 1967) Furthermore it was known that strongly positive pigs were most likely (about 3 1) to be found among younger animals rather than among sows and boars (*Work* 1967)

It was decided therefore to look for a pig with a dye test titre of 1 250 or higher among six months old swine in a slaughter house

Blood samples were collected during slaughtering in the morning and swine and blood samples were marked with identification numbers The serological examination was made on the same day All sera were examined in the Sabin Feldman dye test after inactivation at 56 C for 30 minutes (Results were known eight hours after slaughtering) The pig selected for the present study was collected at the slaughter house on the following morning 24 hours after slaughtering It had been stored overnight at +4 C

Preparation of the Pork

Various cuts of meat were then submitted to different methods of preparation precisely as similar cuts are usually prepared either commercially or in the kitchen All steps were carefully registered with regard to time temperature and salt concentration

Part of each cut about 125 g was left unprepared in the refrigerator at +4 C These parts were used as controls of the presence of viable cysts in the meat prior to preparation

Meat balls were prepared from shoulder and fried for 12 minutes The temperature in the centre rose to 85 C

Pork chops were made from foreloin They were fried for 10 minutes on each side and a temperature of 80 8? C was reached in the centre of the meat

Spare ribs were roasted in the oven for two hour At the beginning the temperature of the oven was ??5 C but after 20 minute it was lowered to 160 C. The temperature in the centre of the meat rose to 85 C

The shank was allowed to simmer in water for two hours and the temperature in the centre reached approximately 100 C

Smoked fillet was prepared from foreloin After having been injected with 2? Bé pickle[1] it was tank-cured in ?? Bé brine for three days at 5 C It was then drained for 24 hours at 5 C and finally smoked for 14 hours at 3 C

Rolled pork was made of the belly It was salted with nitrite salt (NaCl containing 0 6 per cent $NaNO_2$) the amount of salt being 3 per cent of the amount of meat After two days at 5 C it was heated in water at 80 C for 1½ hours followed

[1] Bé (Beaumé) indicates the amount of salt in brine ?? Bé is a brine containing ?0 g of NaCl 0 4–0 9 g of $NaNO_3$ and 5 10 g of $NaNO_2$ per l t

by air cooling at 5° C. The temperature in the centre of the meat during heating was not measured but from experience it can be stated that it was above 70° C.

Prime collar was prepared from the neck and injected with a 22° Bé pickle.

Part of the shoulder was stored in the freezer at —40° C for nine days. Meat balls were prepared from this meat in the same way as above. They were fried for 12 minutes and a temperature of 87° C was reached in the centre.

Corresponding raw and prepared specimens were processed simultaneously, the number of specimens processed per day being 2–4.

Isolation of Toxoplasma Cysts

The peptic digestion technique as developed by *Jacobs & Remington* (1960) was used for isolation of toxoplasma with a slight modification as described in detail in an earlier paper (*Work* 1967).

After digestion the final sediment was inoculated into 10–20 mice dependent on the volume. Four weeks old white mice of Statens Seruminstitut's own breed were used. Each mouse was given 1 ml intraperitoneally. No bacterial growth could be demonstrated by spreading the inocula on blood agar plates.

In every group five not inoculated mice were used as controls of possible spontaneous infection.

The interval from slaughtering to processing of the meat and inoculation of the mice varied from 2–11 days with an average of 6.5 days. During that period the samples were stored at +4° C.

The mice were observed for six weeks. Unstained smears of peritoneal exudate and brain from mice that died during that period were examined microscopically. At the end of the observation period the mice were bled from the tail and the blood samples were examined serologically in the dye test. Finally fresh smears of brains from all mice were examined microscopically for the presence of cysts.

RESULTS

Results of the serological examinations are shown in Table 1. The sera are presented in three groups because they were collected at different times and in two different slaughter houses. Group I and group III were from the same slaughter house.

TABLE 1

Dye Test Titres of 329 Swine

Group number	Number of sera	Negative < 1:10	Positive ≥ 1:10	1:10	1:50	1:250
I	82	76 (92.7 %)	6 (7.3 %)	4 (4.9 %)	2 (2.4 %)	
II	98	89 (90.8 %)	9 (9.2 %)	4 (4.1 %)	5 (5.1 %)	
III	149	125 (83.9 %)	24 (16.1 %)	10 (6.7 %)	13 (8.7 %)	1 (0.7 %)
Total	329	290 (88.1 %)	39 (11.9 %)	18 (5.5 %)	20 (6.1 %)	1 (0.3 %)

Group I was collected in March 1965. Among 82 sera 76 (92.7 per cent) were negative with a titre of < 1:10 in the dye test. Six (7.3 per cent) were positive, four had a titre of 1:10 and two had 1:50.

Group II was obtained in April 1965. Among 98 sera 89 (90.8 per cent) were negative and nine (9.2 per cent) positive. Four had a titre of 1:10 and five 1:50 in the dye test.

Group III was collected in February 1966 In this group 125 (83 9 per cent) of 149 sera were negative 24 sera (16 1 per cent) were positive 10 with a titre of 1 10 13 with 1 50 and one with 1 250 in the dye test

A *total* of 329 sera were examined 290 (88 1 per cent) were negative with a titre of < 1 10 39 (11 9 per cent) were positive 18 had a titre of 1 10 20 had 1 50 and one had 1 250 in the dye test This pig was selected for resistance studies

Isolation of toxoplasma from 16 specimens eight prepared and eight raw was attempted The results are presented in Table 2

TABLE 2

Isolation of Toxoplasma from Raw and Prepared Pork

		No of mice DT pos /inocul		No of mice with cysts/inocul		Control mice positive/ number
		prep	raw	prep	raw	
Shoulder	Meat balls	0/10		0/10		0/5
	Raw		16/16		16/16	0/5
Loin	Chops	0/10		0/10		0/5
	Raw		16/16		16/16	0/5
Ribs	Spare ribs	0/10		0/10		0/5
	Raw		10/10		10/10	0/5
Leg	Boiled	0/10		0/10		0/5
	Raw		9/10		9/10	0/5
Loin	Smoked fillet	0/10		0/10		0/5
	Raw		18/18		18/18	0/5
Belly	Rolled pork	0/10		0/10		0/5
	Raw		10/10		10/10	0/5
Neck	Prime collar	0/20		0/20		0/5
	Raw		10/20		10/20[b]	0/5
Shoulder —40 C 9 days	Meat balls	0/10		0/10		0/5
	Raw		0/10		0/10[d]	0/5
Total		0/90	89/110	0/90	89/110	0/80

Remarks [a] 1 mouse died on the sixth day (no toxoplasma found on microscopy) [b] 8 died 1–5th day (no toxoplasma found on microscopy) 2 died on the 27th day (not examined) [c] mice died 30th day (no toxoplasma found on microscopy) [d] 1 mouse died 35th day (no toxoplasma found on microscopy)

A total of 90 mice were inoculated with digests of prepared meat Two mice died during the observation period *viz* on the 30th day after inoculation No toxoplasma cysts were found by microscopy of fresh smears from their brains The remaining 88 mice were examined serologically at the end of six weeks None of these had developed toxoplasma antibodies Fresh smears of their brains were examined microscopically and no cysts were found

110 mice were inoculated with digests of raw meat. Twelve died during the observation period. Unfortunately two of these were not examined. Out of the remaining 10 nine died in the course of 1–6 days after inoculation. They contained very little or no peritoneal exudate and no toxoplasma was found by microscopy. One mouse died on the 35th day after inoculation and no cysts were found at microscopy of its brain. 98 mice were examined serologically in the dye test at the end of six weeks. 89 of these were positive. Fresh smears of their brains were examined microscopically and toxoplasma cysts were demonstrated in the serologically positive mice. Nine mice did not develop antibodies and no cysts were found in their brains. They had been inoculated with digests of meat that had been stored at –40 C for nine days.

A total of 80 control mice were examined at the end of the observation period. None of these had developed toxoplasma antibodies and no cysts were found by microscopy of fresh smears of their brains.

One additional experiment was performed. A whole leg was prepared as smoked ham. It was tank cured in 22 Be brine at 5 C for two weeks. Afterwards it was drained at 5 C for 6 days and finally it was smoked at 20–22 C for 5 days. Isolation of toxoplasma was attempted as described above. 14 mice were inoculated but none of these became positive. Since the preparation of the meat lasted for 25 days, it was impossible to store the control cut at +4 C for the same period and therefore this experiment is not included in Table 2.

DISCUSSION

The serological examinations were performed merely to find a pig to be used in the resistance studies and no serological survey was intended. In the present material the percentage of serologically positive individuals varied from 7.3 in group I to 16.1 in group III. In evaluating these figures it must be remembered that the rate of infection is very high on some farms while low on others. Consequently there will be great day to day variations in the number of serologically positive animals brought in for slaughtering. To obtain a true impression of the average infection rate in a certain area it would probably be necessary to examine the livestock of every single farm.

The results obtained in the isolation studies confirm those found in our former studies of encysted toxoplasma in meat (*Work* 1967). Toxoplasma could be isolated from all raw samples, thus indicating that consumption and handling of raw meat involve a risk of infection. On the other hand no parasites could be isolated from any prepared sample. Meat balls, pork chops, spare ribs, shank and rolled pork were heated and the temperature in the centre of these samples rose to 70 C or higher, a temperature which is generally accepted as sufficient to kill toxoplasma.

Nor was it possible to isolate toxoplasma from smoked fillet and prime collar Both had been salted and the fillet had also been smoked The killing of parasites in these cases as well as in smoked ham might be due to changes in osmotic pressure

As already pointed out the different cuts of meat were prepared as they are usually prepared before consumption It might be interesting deliberately to undercook the meat in order to see at which temperature the killing of parasites actually takes place

Only one of the samples was frozen It was stored at —40 C for nine days and no organisms could be isolated afterwards A temperature of —40 C is considered ideal for storage of meat because all enzymatic processes are stopped Commercially however long time storage of meat takes place at —25 to —30 C and short time storage at —18 to —20 C Further studies should probably be concentrated on these temperatures

Preliminary experiments in this laboratory seem to indicate that even 24 hours at —20 C are sufficient to kill encysted toxoplasma in meat However the material is too small to permit conclusions to be drawn and the problem will be investigated in a separate study

SUMMARY

The resistance of toxoplasma encysted in pork is rather low and apparently comparable with that of isolated cysts Even gentle preparatory measures such as slight salting and low temperature smoking are sufficient to kill the parasites in smoked fillet Normal household procedures such as frying roasting or cooking also seem to eliminate encysted toxoplasma

In the kitchen the risk of becoming infected with toxoplasma is apparently related exclusively to raw meat

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ON THE NATURE OF THE MU FACTOR

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Investigations concerning the episome like Mu factor of *E coli* (11) have revealed several similarities with the R factors (21) When a bacterial cell acquires the Mu factor it becomes resistant to low levels of streptomycin (8 9) and such low level resistant cells have been shown to carry the Mu factor in an extrachromosomal state (11) Approximately 10⁴ of the cells in such a population mutate to a higher level of streptomycin resistance This phenomenon which certainly is no mutation in the conventional sense originated the designation Mu factor In the cells with a higher level of streptomycin resistance i e in the Mu str r state the bacterial chromosome is modified the genetic determinant for the higher level resistance is located on the bacterial chromosome near the methionine region (7) its location as well as its physiological character being definitely distinct from that of ordinary streptomycin resistance (7 10) Although the R factor has been reported to modify the bacterial chromosome (6) the mechanism as well as the resulting level of resistance seems entirely different from those of the Mu factor

The Mu str r state was thought tentatively to be the result of an attachment to or an integration of the Mu factor in the bacterial chromosome This would then imply that the Mu factor is a true episome It is currently believed that the integrated and the autonomous state of episomes are mutually exclusive (13) since this seemed to be the case both with the F factor and the temperate phages upon whose common behaviour the concept of the episomes was based The Mu factor however *still seems to be carried in an extrachromosomal infectious form* even when the bacterial chromosome is modified as a result of its integration or attachment A somewhat similar situation has also been described for the variant sex factor the F or F factors (3) where attachment of the factor to the bacterial chromosome does not affect the maintainance and free transmissibility of the autonomous population

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the method described by Weissbach Lipton & Lisio (26) or by the method of Marmur (17)

Preparation of ¹⁴C-DNA ¹⁴C-DNA was prepared essentially as described for H³-DNA with minor modifications The thymine concentration was 0 02 mM and 0.3 mC per litre medium All ¹⁴C-DNA was extracted by the method of Marmur (17)

Density gradient centrifugations were carried out essentially as described by Jyssum & Jyssum (14) Approximately 50 µg of DNA was centrifuged in a total volume of 5 0 ml The centrifugation was performed in a solution of the following composition 4 704 g CsCl (Analar Hopkin and Williams Ltd Essex England) 100 µl DNA and NaCl citrate buffer pH 7 4 (NaCl, 0 14 M Na$_3$citrate 0 015 M) to volume This will result in a final density very close to 1 710 g/cm³ The samples were centrifuged in a Spinco model L centrifuge at 30 000 R.P M for 72 hr at 15 C, using a SW 39L head. The samples were fractionated by puncturing the tube and collecting 6 drops per fraction The first 150 and the last 180–200 drops were collected separately when it was found that they never contained radioactive material All samples were counted in a Packard Tri Carb liquid scintillation spectrometer The scintillation solution contained 1925 ml of xylene 1925 ml of dioxan 1150 ml of ethanol 400 g of naphthalene 25 g of PPO and 250 mg of POPOP (9) A precipitate was formed when the scintillation fluid was added to the fractions but this was found not to interfere with the countings

Conjugation technique Donor and recipient were pregrown to logarithmic phase at 37 C with shaking as a rule in complete medium Equal volumes of donor and recipient cultures were mixed diluted with fresh medium if necessary and incubated at 37 C without shaking Samples were withdrawn at various times diluted in saline and shaken vigourously by means of a Vortex Junior Mixer for 1 min Proper dilutions were then spread on selective media

RESULTS

Transfer of Low Level Resistance to Streptomycin (Mu Factor) from Donor to Recipient in a K12 System

The kinetics of transfer of the Mu factor in a K12 system is seen from Fig 1 The curves are identical whether the donor is of the low level resistant (Mu) or the higher level resistant (Mu str r) type The factor is transferred with equal ease whether the pregrowth and conjugation are taking place in a minimal medium or in complete medium The kinetics of transfer of the Mu factor to an Hfr strain is complicated by the fact that genetic material is donated in both directions thus rendering the results less clear cut There is however no difficulty in transferring the factor to F or Hfr strains It has been reported earlier (11) that the transfer of the Mu factor was enhanced by the presence of the F factor in the donor This point has been reinvestigated in the K12 system using our present improved technique of selection No enhancement was found in transfer when the donor was F or Hfr

In the present system of conjugation and selection no time is allowed for phenotypic expression (8) It has been ascertained that the number of recombinants is the same whether streptomycin is added to the selective medium immediately (the final concentration being 15 µg per ml of minimal medium) or after a delay of up till 6 hours

Attempts to transfer the Mu factor by means of conjugation or mixed cultivation to strains of *Proteus vulgaris Proteus mirabilis Proteus rettgeri* or *Proteus morgani* as well as to strains of *Serratia*

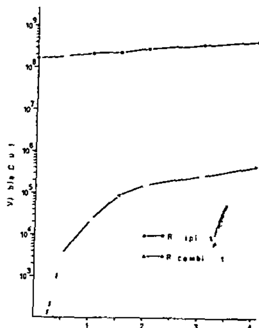


Fig 1

Kinetics of the transfer of the Mu factor in a K12 system D nor T71 Mu thr his Recipient T71 met The cultures were pregrown in Heart Infusion Broth mixed and incubated at 37 C. Samples were taken at intervals and assayed for viable cells on minimal agar plates supplemented with methionine (for the recipient) and methionine and streptomycin (15 μg per ml) (for the "recombinants")

marcescens have failed under the conditions used Whether the negative results are due to lack of transfer or to a lack of expression is not known It has been reported (20) that the R factor is inhibited in its expression in *Serratia marcescens* by the presence of streptomycin

Transfer of the Mu factor from a K12 donor to *E coli* 15 T took place with very low frequency After mixed incubation overnight 10 of the recipients had received the factor as compared to 10⁻¹–10⁻² in a K12 system under similar conditions Also transfer of the factor between mutants of *E coli* 15 T was equally slow

Attempts to transfer the Mu factor by mixed cultivation to the rec mutants KMBL 239 240 241 and 243 as well as to their rec parent strain KMBL 146 have been futile

Interactions between the Mu Factor and the F Factor in E coli K12

Quite early it was reported that R factors interfere with the expression of the F factor when both factors are present in the same cell (23 24 12) Later two types of naturally occurring R factors have been found which do or do not interfere with the F factor respectively (25)

At the time of the earliest reports of this interference it had been found that the Mu factor suppressed the donation of genetic material in an Hfr strain Since the phenomenon thus seems to be another characteristic common for these two types of factors some investigation was carried out It can be seen from Table 1 that the sensitivity to the male specific bacteriophages f and MS2 is completely blocked in Hfr or F^+ strains which carry the Mu factor whereas the sensitivity to the arbitrarily chosen phage P1kc is unaffected

TABLE 1

Suppression of Sensitivity to Male Specific Bacteriophages in Hfr or F Strains of E coli *K12 Carrying the Mu Factor*

Strain tested	Sensitivity to the bacteriophages f	MS2	P1kc
Hfr Cavalli	+	+	+
Hfr Cavalli Mu	—	—	+
Hfr Cavalli try	+	+	+
Hfr Cavalli try Mu	—	—	+
Hfr Hayes	+	+	+
Hfr Hayes Mu	—	—	+
Hfr P10	+	+	
Hfr P10 Mu	—	—	
Hfr P13	+	+	
Hfr P13 Mu	—	—	
K12 W6 F	+	+	
K12 W6 F Mu	—	—	

In crosses Hfr Mu × F the recombination frequency is reduced to approximately 10^{-2} of the frequency in a comparable Hfr × F cross Also crosses Hfr × Hfr Mu indicate that Hfr Mu actually acts like an F recipient in crosses with ordinary Hfr strains The Mu factor thus seems to be like the R factors of the fi^+ type

The Absence of Chromosomal Transfer by the Mu Factor

Several of the bacterial episomes have the ability to effect transfer of genetic information from their hosts and this ability is a very important property of episomic elements *Watanabe* (21) reports that his group has been unable to obtain evidence that R factors make F strains of *E coli* K12 fertile whereas *Sugino & Hirota* (19) reported recombinations to take place with frequencies ranging from 10^{-6} to 10^{-8} *per donor cell in an ordinary* R F × R F *cross In a specific F* derivative of an Hfr strain (cited 21 p 103) R mating occurred with high frequency ranging from 10^{-4} to 10^{-6} per donor cell

Crosses have been performed using F Mu or F Mu str r as donors of chromosomal markers and mutants of the ordinary F strain *E coli* K12 T71 as recipients The following mutants have been tested as re

cipients in these crosses T71 met T71 arg T71 gly/ser T71 try T71 lys T71 nic and T71 pro As possible donors were used T71 Mu thr his T71 Mu str r thr his as well as T71 thr his Mu⁻ in control systems Under the conditions used there is no evidence of any transfer of chromosomal markers mediated by the Mu factor

Evidence for the DNA Nature of the Mu Factor

The acquisition of cytoplasmic particles by bacteria has in many cases been shown to be correlated with an increase of the DNA content of the cells from 3 per cent up to more than 10 per cent In some cases the base composition of this foreign DNA differs from that of the chromosomal DNA of the host (27 5 18) By means of ³²P decay experiments the nature as well as the relative size of the F_1 and F particles have been estimated 1 3–3 7 per cent and 2 8–8 1 per cent of the bacterial nucleus respectively the upper limit being the most probable (4)

TABLE 2

Relation between the DNA and Protein Content in Exponentially Growing Cultures of E coli with and without the Mu Factor

Strain employed	µg DNA per µg of protein (Average)	DNA/protein calc in per cent
E coli K12 T71	0 02372	100
E coli K12 T71 Mu	0 02675†	112 8
E coli K12 T71 Mu str r	0 02593†	109 3
E coli 15 T	0 01609§	100
E coli 15 T Mu	0 01962§	122 5
E coli 15 T Mu str r	0 02274§	141 4

Average of 10 separate experiments
† Average of 3 separate experiments
§ Average of 6 separate experiments

Jyssum & Jyssum (14) taking advantage of the proportionality between the content of bacterial DNA and proteins under all conditions of growth (16) found the amount of DNA per unit protein to be 15 per cent higher in cultures of exponentially growing cp Neisseria meningitidis than in the corresponding cp variants Analyses were therefore performed in this present system to elucidate whether the presence of the Mu factor in a population of bacterial cells was followed by any measurable increase in the DNA/protein ratio as compared to a wild type control Results from such experiments are recorded in Table 2 It will be seen from this table that there is a concomitant increase in the DNA/protein ratio of approximately 10 per cent when the Mu factor is introduced into an *E coli* K12 strain the ratio being the same for the Mu cells as for the Mu str r cells The slight difference recorded may not be significant it may be within the error of the method When the Mu factor is introduced into *E coli* 15 T which initially

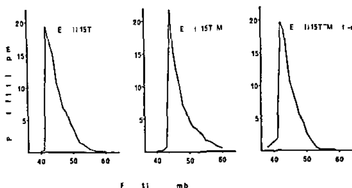


Fig 2

Distribution of DNA in a CsCl density gradient from three variants of *E coli* 15 T⁻ The curves show counts per minute of H^3 labelled DNA per fraction plotted as per cent of the total number of counts against fraction number

has a considerably lower DNA/protein ratio (although the growth rate for K12 T71 and *E coli* 15 T⁻ in this medium is the same with a gene ration time of some 27 min) one finds an increase in this ratio of some 20 per cent Upon isolation of the higher level streptomycin resistant variant one finds another increase of some 20 per cent in the ratio to approximately 140 per cent of the wild type ratio

Attempts to Isolate the Extra DNA by Means of Density Gradient Centrifugation

The results presented here are from three DNA preparations *E coli* 15 T⁻ *E coli* 15 T Mu and *E coli* 15 T Mu str r Fig 2 shows the di stribution of the DNA in the gradients The curves have been trans formed into per cent distribution of the total number of counts per minute within the fractions recorded No significant difference can be observed in the distribution of the DNA from these preparations No separate peak is found in the preparations from *E coli* 15 T Mu or *E coli* 15 T Mu str r although particularly in the latter strain a very high percentage of the DNA must be foreign

If the extra DNA really has the same density as the native *E coli* 15 T DNA one should expect to find identical curves when using a double labelling technique centrifuging two differently labelled DNA preparations together and then calculating the distribution of the ^{14}C and ^{3}H respectively By employing this technique one should avoid the variance introduced by the handling of the tubes after the centrifuga tion as well as the variance invariably introduced by the fractionation The strains *E coli* 15 T and *E coli* 15 T Mu str r were chosen for such experiments because of the greater difference in the DNA con tent For technical reasons it is convenient to have a much higher pro portion of H than C in a double lable experiment In order to avoid

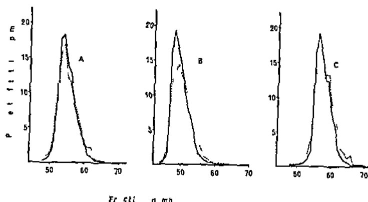


Fig 3

Distribution of DNA in a CsCl density gradient Two DNA preparations differently labelled are centrifuged together The solid line shows the ^{14}C distribution the dotted line shows the H^3 distribution

A ^{14}C DNA from *E coli* 15 T and H^3 DNA from the same organism (control run)
B ^{14}C DNA from *E coli* 15 T and H^3 DNA from *E coli* 15 T Mu str r
C ^{14}C DNA from *E coli* 15 T Mu str r and H^3 DNA from *E coli* 15 T

The curves show counts per minute per fraction plotted as per cent of the total number of counts against fraction number

any artifacts due to difference in bulk ^{14}C–DNA and H^3–DNA were prepared from each of the strains Typical curves from such density gradient centrifugations are seen in Fig 3 From the control experiment (Fig 3A) it can be seen that when the ^{14}C–DNA and the H^3–DNA originate from the same strain the wild type E coli 15 T in this case the resulting distributions of H^3 and ^{14}C are virtually identical When the different labelling originates from different mutants however a certain bipartite distribution of the H^3 is seen This bipartite distribution is seen regardless of whether the H^3–DNA originates from the wild type (Fig 3C) or from E coli 15 T Mu str r (Fig 3B) This is in contrast to the unimodal distribution one finds when each DNA preparation is run separately as well as to the control experiment with double labelling originating from the same mutant

DISCUSSION

The Mu factor was isolated in this laboratory in 1960 as a part of a search for bacterial strains with different types of genetic instability One strain of *E coli* 63a was found to exhibit an extreme mutability to streptomycin resistance and this mutability was found to be due to the presence of a transmissible episome like factor the Mu factor (11) We were looking for genetic instability and found what was thought to be a genetic instability At this time the discovery of the resistance transfer factors was reported in international journals (22) The continued work with the Mu factor in this laboratory as well as the

work with the R factors in other laboratories has revealed an increasing number of similarities between these factors

The transfer of the Mu factor from donor to recipient in a K12 system is somewhat slower but very similar to the transfer of the factor R(Sm Cm Tc Su) (22) When introduced into a K12 strain this R factor gives a level of streptomycin resistance similar to that of the Mu factor namely between 10 and 25 µg streptomycin per ml (22 9) When the R factor is introduced into a sensitive recipient it is reported to need a lag of some 90 min before streptomycin can be added to the selective medium whereas the Mu factor requires no such lag This lag is reported to be different from the lag of the true streptomycin resistance which is the phenotypic lag for segregation and expression of a recessive marker the lag of the R factor may be less than one generation time This difference between the two factors may be an apparent one due to the difference in the selection technique The lag of the R factor is probably just a need for a physiological adjustment which in some cases also is necessary for the Mu factor (9) Whereas the R factors are reported to be transferred with ease to other strains of *E coli* as well as to other species of bacteria the Mu factor is very selective In its interactions with the F factor the Mu factor resembles the R factors of the fi type

In the present investigation no transfer of chromosomal markers from the host cells mediated by the Mu factor could be observed neither from cells in the Mu nor in the Mu str r state *Watanabe* (21) reports that his group could not obtain evidence of any R mediated chromosomal transfer but *Sugino & Hirota* (19) report such chromosomal transfer to take place with extremely low frequencies

When a K12 strain acquires the Mu factor its DNA content is increased by some 10 per cent This implies that the Mu factor consists of DNA It says however nothing about the size of the Mu factor since nothing is known about the number of particles present per chromosome In the higher level streptomycin resistant cells (the Mu str r state) the chromosome is modified since the higher level resistance segregates like a chromosomal marker located in the met–region of the bacterial chromosome (7) That the bacterial cells in which the chromosome is modified by the attachment or integration of the Mu factor still possess the Mu factor in an infectious form is known from two types of experiments Firstly in this present investigation it has been found that the kinetics of transfer of the Mu factor to a sensitive recipient is the same whether the donor is Mu or Mu str r Also when Mu str r cells are crossed with Hfr Cavalli either str s or str r the streptomycin sensitive progeny all possess the Mu factor in its extrachromosomal state (7) Thus the progeny strictly speaking is not streptomycin sensitive but low level resistant One might therefore think that the Mu str r cells possess at least two Mu particles per chromosome Since the amount of extra DNA is not more but if anything rather less

in K12 Mu str r than in K12 Mu one might hypothesize that also these have at least two particles per chromosome

E coli 15 T shows an interesting phenomenon Firstly the ratio DNA/protein in the wild type is considerably lower than that in the K12 wild type growing under similar conditions the growth rate of the two strains being the same in the medium used The reason for this is not known Secondly the extra DNA introduced by the Mu factor increases the DNA/protein ratio by some 20 per cent And when the cells are in the higher level resistant state the increase approximately doubles to a DNA/protein ratio 140 per cent of that in the wild type *E coli* 15 T⁻ One might think that if there were one copy of the Mu factor per chromosome in the Mu state there might be two in the Mu str r state one attached to the chromosome and one extrachromosomal

Analyses by density gradient centrifugation might be expected to reveal something about the molecular nature of the Mu factor If the base composition is significantly different from that of the chromosomal DNA this might be indicated The gradient analyses give no indication of any heterogeneity of the DNA of the three preparations (Fig 2) We have pointed out before similarities between the Mu factor and the R factors of the fi type Looking at the results of *Falkow Citarella, Wohlhieter & Watanabe* (5) where they report the localization of the different drug resistance determinants within the density region of the R factor DNA they find that the determinant for Sm resistance as well as those for Tc Su and Kanamycin have a density very close to that typical of E coli DNA whereas the determinant for Cm resistance seems to be of a higher specific density According to our results it is thus quite plausible that the Mu factor really is an R factor carrying resistance to streptomycin only The fi determinant was initially assumed to be connected with the Cm marker since no naturally occurring fi R factors were seen to carry the Cm resistance *Falkow et al* (5) report however that the factor R(Su Sm Tc) isolated by *Naomi Datta* (cited 5) is of the fi type but shows no band of specific density 1 716 g/cm^3 (corresponding to the Cm marker) If one considers the Mu factor to be an R factor of the fi⁺ type our results are consistent with this finding by Datta concerning the location of the fi determinant in the density band of course with due regard to the limitation of our method

The characteristic feature of the Mu factor which initially roused our interest in it originated its name and which we thought to be unique may not be unique but perhaps a common feature of several naturally occurring R factors *Aandahl* (personal communication) has isolated several R factors from hospital strains of enterobacteria R factors with various resistance determinants All of these R factors which carry resistance to streptomycin show a similar phenomenon

In the experiments with differently labelled DNA originating from

two different organisms *E coli* 15 T and its Mu str r variant the distribution of the H^3 counts was somewhat bipartite (Fig 3) definitely different from the control run (Fig 3A) where both H^3 and ^{14}C DNA originated from the same organism (*E coli* 15 T) as well as from the distribution when the DNAs are run separately (Fig 2) The reason for this decrease in H^3 counts is not at all clear but it looks like an artefact perhaps a specific quenching of the H^3 radiation In order to explain the phenomenon the following hypothesis was suggested If the higher level resistance is the result of an attachment to or an integration of the Mu factor in the bacterial chromosome one has to anticipate homologous structures in the two DNAs Homologous structures probably have the same specific density If one could conceive that these homologous structures would aggregate in some way during the centrifugation this might be thought to result in a reduction of the number of disintegrations recorded from the tritium because of the low energy of its radiations The recording of the ^{14}C disintegrations which have a much higher energy might well be unimpaired Such an aggregation is however difficult to visualize chemically even with a preceding separation of the two DNA strands

The results of these present experiments seem to suggest that the Mu factor has a specific density which makes its DNA sediment together with the bulk of the *E coli* DNA and they may also suggest the existence of homologous structures

SUMMARY

Evidence has been presented suggesting that the episome like Mu factor is an R factor of the fi^+ type carrying resistance to streptomycin only The Mu factor does not seem to mediate transfer of chromosomal markers from its host neither when it is extrachromosomal nor when it is in its possibly integrated state

Like other R factors the Mu factor seems to be of DNA nature When present in K12 the Mu factor may exist in at least two copies per chromosome regardless whether it is extrachromosomal or possibly integrated In *E coli* 15 however it may be present in only one copy per chromosome when extrachromosomal but at least in two copies *when it is also attached to or integrated in the chromosome* This finding is consistent with the fact that cells which carry the Mu factor attached to or integrated in the chromosome also carry the factor in its infectious form

Density gradient centrifugations indicate a base composition similar to that of *E coli* DNA The existence of homologous structures in the Mu factor DNA and the *E coli* DNA is suggested supporting the hypothesis of the Mu factor being a true episome

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STUDIES ON IMMUNOLOGICAL TOLERANCE TO LCM VIRUS

9. *Induction of Immunological Tolerance to the Virus in the Adult Mouse*

By

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It has always been difficult to induce immunological tolerance to living or self reproducing antigens in the adult mouse. This has been carried out more successful with polysaccharide antigens and protein antigens as in Felton's immune paralysis (4) or Dresser's protein overloading (2, 3). Using LCM virus as antigen temporary states of complete and incomplete tolerance have been induced in the adult mouse by virus inoculation in connection with treatment with antimetabolic drugs, x-ray treatment and cortisone treatment (7, 9). It has recently been possible to induce such a tolerance in the mouse by virus inoculation in connection with treatment with antilymphocytic serum (5, 11).

With the LCM virus strain used in this laboratory intracerebral inoculation of adequate doses of the virus into adult mice usually resulted in the death of the animals from LCM disease. Unlikely large doses could be inoculated *intraperitoneally* in the mice used with only little lethality, but these always resulted in the development of an immune state in the animal, never in a tolerant state (17).

In the following a report will be given of the results of experiments in which adult mice were inoculated with large doses of LCM virus resulting in the development of a tolerant state in the surviving mice.

MATERIALS AND METHODS

The mice used were from a highly inbred strain of C_3H mice which has been used in this laboratory for several years. The normal animals have never been in contact with LCM virus.

The LCM virus used was our usual LCM strain. The stock suspension usually consists of a ten per cent homogenate of spleens harvested from adult C_3H mice six or seven days after intraperitoneal virus inoculation. *The medium used con*

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sists of PBS without Ca and Mg^{++} containing ten per cent horse serum and one per cent penicillin/streptomycin solution (20 000 i u /ml penicillin and 5 mg/ml streptomycin) In addition in the present experiments with inoculation of large dosages of the virus the suspension was made from spleens of C_3H virus carrier mice The virus suspension was kept at —70 C until use

The virus titrations were carried out by intracerebral inoculation of ordinary white Swiss mice of decimal dilutions of whole blood the lowest dilution being 10 = *undiluted blood (15) The titration end points were calculated according to Karber s* method (10)

The complement fixing antigens used were prepared from spleens of virus carrier mice and made by an acetone extraction method described by *Grešikova & Casals* (6) and by *Clarke & Casals* (1) The procedures used in the complement fixation test were tho e described in a previous paper (18)

EXPERIMENTAL

Intraperitoneal Inoculation of a Standard Dose of LCM Virus into Adult Mice

Reference will first be made to the results of intraperitoneal inoculation of 3 000 I D_{50} LCM virus into adult female C_3H mice This virus dosage has been used for several years as a standard vaccination dosage for adult C_3H mice Given intraperitoneally it very seldom results in any lethality In some animals inoculated in this manner only slight temporary illness can be observed

A group of 42 adult female C_3H mice three to four months old weighing about 20 g were inoculated on day 0 with 3 000 LD_{50} LCM virus intraperitoneally For the next 50 days two to seven animals from this group were sacrified at intervals their blood was titrated for virus content and their serum for the amount of complement fixing antibodies In Fig 1 these observations are shown graphically Initially high virus titres are seen They decrease in most of the animals after a week after that time all the titres are < 10 The complement fixing antibodies appear after a week and reach titres of 64 to 128 In

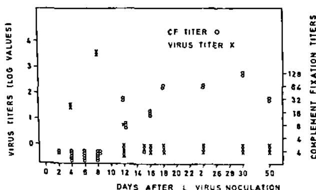


Fig 1

Virus titre in the blood and complement fixing antibody titre from 42 adult, female C_3H mice sacrified and titrated at various days aft r intraperitoneal inoculation of 3 000 LD LCM i us

other groups of mice not followed systematically this has also been found to be the case when the animals are tested more than one year after the virus inoculation No tolerant state has ever been found in hundreds of mice thus treated They become highly immune and repeated inoculations of the virus in the above mentioned dosages do not alter the state not even as regards antibody titres

Intraperitoneal Inoculation of Large Doses of LCM Virus into Adult, C_3H Mice

In the following experiment a group of 30 normal (unsensitized) adult female C_3H mice of an age of three months weighing about 20 g were each inoculated intraperitoneally with an LCM virus dose of 170 000 LD_{50} on days 0 2 5 7 and 9 Six mice died during the inoculation period During this and in the following period all the animals were ill In all cases the pelt was untidy they lost in weight showed reduced motility and some had diarrhoea In the following time blood samples were taken from the inner canthus of the eye at intervals and titrated individually for virus content and for complement fixing antibodies Only eleven mice survived 150 days after the last virus inoculation They had all virus titres of $\geqq 10^{4.5}$ in the blood and complement fixing antibody titres of 128 and 256 Two of these mice died 247 days after the last virus inoculation the remainder of the group was followed for 360 and 420 days after the last virus inoculation

On the basis of the course of the virus titres and the complement fixing titres in the individual mouse among the eleven survivors three types of course could be distinguished after this long observation period These three types are shown in the Figs 2 to 4 These graphs show the course in individual mice representative for the type Minor variations from the course shown occurred in some of the members of each group but these will be described in the text

In three of the mice the course was of type A see Fig 2 In all three mice the courses of the complement fixing antibodies were nearly identical with almost constant values of 128 to 512 for a long time finally decreasing to a titre of <4 i e negative complement fixing titre The virus titres were quite high in all three animals for the first weeks and then decreased to a low level which in two mice lasted for two or three months with titres $< 10^{0.5}$ and for the third mouse of this type lasted for only two weeks with a minimum titre of $10^{1.8}$ The final result however for all three mice of this type was that for the last months of the observation period they had virus titres of about 10^{4} and no complement fixing antibodies i e a completely tolerant stat to the virus

In six of the eleven mice the course was of type B see Fig 3 In all six mice the course of the complement fixing antibodies were nearly identical with titres at a rather constant level (titres of 128 to 512) for

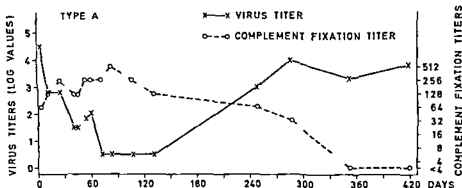


Fig 2

The course of the virus titre in the blood and the complement fixing antibody titre from a single normal adult female C H mouse which had received a large dose of LCM virus intraperitoneally (5 × 170 000 LD) Day 0 is the day of the last virus inoculation With reference to the type see text

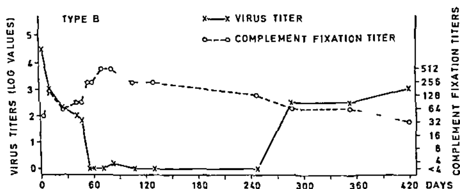


Fig 3

The course of the virus titre in the blood and the complement fixing antibody titre from a single normal adult female C_3H mouse which had received a large dose of LCM virus intraperitoneally From the same group of surviving mice as in Fig 2

most of the observation period but during the last months these decreased although they did not disappear as in type A The final values were two mice had a titre of 8 one a titre of 16 one a titre of 32 and two a titre of 64 The virus titres in this type started as was the case in type A at a high level for the first weeks they then decreased to the low level In four of the six mice this low level lasted for about six months and all titres in this period were $< 10^{0.5}$ In two mice the low level lasted for only about two to three weeks one had a minimum titre of < 10 and one a minimum titre of 10^{0} In all six mice the virus titres gradually increased again to titres $> 10^{-3}$ The final result for these six mice after the long observation period was that they all had virus titres at tolerant virus carrier level *i e* $> 10^{-3}$ for the last

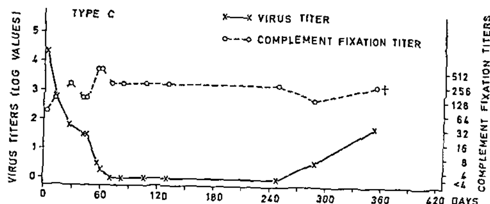


Fig 4

The course of the virus titre in the blood and the complement fixing antibody titre from a single normal adult female C_3H mouse which had received a large dose of *LCM virus intraperitoneally* From the same group of surviving mice as in Figs 2 and 3

months and low to moderate complement fixing titres This indicates an incompletely tolerant state

In the last two mice of the eleven survivors the course of the viraemia and antibody titres was of type C see Fig 4 In both mice the antibodies maintained almost constant titres during the whole observation period The final titres were 128 and 256 Initially as in the other two types the viraemia started with quite high titres during the first weeks it then decreased to a low level with titres of < 10 and finally increased to titres of $10^{0.8}$ and $10^{1.8}$ respectively *i e* an incompletely tolerant state The observation periods for these two mice were somewhat shorter than for the other mice They died 246 and 360 days after the last virus inoculation

Table 1 contains a review of the eleven mice with regard to the distribution according to type and the duration of the low level period

TABLE 1

Review of the Distribution of a Group of Adult Mice Surviving Intraperitoneal Inoculation of Large Dosages of LCM Virus According to Type (see Text)

Type	Number of mice	Number of mice with low virus level for 2 weeks	Number of mice with low virus level for 2 to 6 months
A	3	1	2
B	6	2	4
C	2	–	2

DISCUSSION

As stated above an LCM virus dosage of 3 000 LD₅₀ given intraperitoneally into adult mice resulted in the development of an immune state in

the animals and never in the development of a tolerant state The high doses of virus used in the second of the experiments reported here are extremely high but nevertheless resulted in the development of a tolerant state in the surviving animals

During the development of this tolerance several very interesting immunological phenomena were observed during the very long observation period necessary

In all mice surviving the virus inoculations very high virus titres were found in the blood (> $10^{4.5}$) during the first week after the inoculations As pointed out previously in connection with the induction of tolerance by inoculation of newborn mice initially high virus titres are necessary for a certain period in order to permit the development of tolerance (8 17) In the present experiments this high virus level was present during the first week after the inoculations and this might be the reason for the later development of the tolerance Many authors consider that the reason why it is so difficult to induce tolerance in the adult animal is that in these animals the immune response develops very soon after the stimulation and tends to reduce the antigen content below a definite threshold (14) In the present experiments an immune response as judged by the presence of complement fixing antibodies of moderate titres developed fairly rapidly and at the same time as the high virus titres Later however the virus titres in the blood decreased to a low level in most of the animals which survived This seems to be the normal reaction usually seen after intraperitoneal inoculation of LCM virus in lower dosages in an adult mouse Presumably the virus was still present in the animals during this low level period In some mice there still were low virus titres It has been shown that trace amounts of virus in the blood may occasionally be found in immune mice and in many mice of this category the virus content of the kidneys was high indicating that the kidneys presumably functioned as a virus reservoir (16) It is apparent that this low level period lasted for several months in most of the animals (Table 1)

After this low level period there is the most interesting occurrence namely that the virus titres spontaneously increase and somewhat later the complement fixing antibodies decrease or disappear Possibly no real difference exists between the three types described If the observation period had been long enough for the types B and C these might have been found to be identical to type A The virus titres increase in most of the animals to constant values > 10^{3} *i e* tolerant virus carrier values (7 8) In type A where in addition the complement fixing antibodies completely disappear the result must be considered a completely tolerant state In type B where the virus titres are at tolerance level but an immune reaction is still present as judged by the presence of complement fixing antibodies the result at this point of time must be considered an incompletely tolerant state This is also the case for type C

To summarize the course is as follows A phase with high viraemia (little or no virus elimination) together with a fairly strong humoral immune reaction (complement fixing antibodies present) A second phase with low or no viraemia (virus elimination present) together with the presence of complement fixing antibodies Hereafter a switch over to the third phase with complete/incomplete tolerance with no virus elimination and no (or little) humoral immune response

As described previously (8) where the LCM virus in mice is concerned the virus elimination (here defined as the elimination of the viraemia) and the humoral immune response (only complement fixing antibodies are measured) are two immune reactions which often run synchronously but may run independently of one another as dissociated immune functions This dissociation is also illustrated in the experiments reported here

It may be assumed that the kinetics of the course reported are as follows The five virus inoculations do not result in homologous interference or cause an immediate immune paralysis because quite high antibody titres are observed shortly after the last virus inoculation together with high viraemia This also means that the virus elimination mechanism either does not function or functions only to a minor degree Previous results and results which are yet to be published indicate that the virus elimination mechanism for LCM virus in the mouse is an immune cellular function which shows only a limited degree of dependence on a humoral immune response This means that in the first phase the immune cellular function is suppressed temporarily while the humoral immune response is normal In the second phase there are both a cellular and humoral response The humoral response is a continuation of that from the first phase but the cellular response begins at this stage presumably caused in normal way by antigen stimulation although it is delayed *i e* the suppression of the cellular response is diminished The switch over to the third phase means that a suppression of the humoral response (decrease or disappearance of the complement fixing antibodies) and the cellular response (no virus elimination) sets in at this point of time—this occurs without any known stimulation (spontaneously) In the individual mouse there is obviously a definite mechanism which controls the degree of immuno suppression in the animal It might be possible to point out features identical to those found by Rowley and Fitch (12 13) in another tolerance system but further explanations will be put forward in a later report containing further facts on this subject

To the author the most interesting and inexplicable phenomenon observed is the late and spontaneous switch over from the second low level phase where both immune functions are working intensely at the same time to the third phase in which these immune reactions are suppressed with the resultant tolerant state What causes this spontaneous switch over is unknown It is remarkable that it appears so

late As it has never been seen in mice inoculated with smaller virus doses it might mean that the first high virus phase has influenced the immune mechanism or its regulation in such a way that an immuno suppression can later develop spontaneously Maximal specific immuno suppression must mean a completely tolerant state and vice versa (7)

These experiments do not at present yield more information about such control mechanism of the immune response to LCM virus in the mouse Further explanations would be philosophy and are omitted until more facts about the problem are available

SUMMARY

A group of 30 normal adult C_3H mice were each inoculated intra peritoneally on five occasions with a dose of 170 000 LD_{50} of LCM virus Eleven mice which survived the last virus inoculation for 150 days were followed for more than one year Blood samples were taken at intervals and titrated for virus content and complement fixing anti bodies From the course of these values in the individual mouse it was possible to distinguish three types of course Initially there were high virus titres and moderate complement fixing titres in all animals In most of the animals the viraemia decreased to a low level for two weeks to six months Thereafter there was a spontaneous switch over to viraemia at tolerant virus carrier level simultaneously with the dis appearance or decrease of the complement fixing antibodies resulting in a tolerant state

It is not possible to give any explanation of this late switch over but the problem is discussed in the light of the assumption of complete tolerance as a maximal degree of specific immunosuppression More evidence supporting the finding described previously that the immune response to LCM virus in the mouse at least has two separate modes of presentation a cellular causing virus elimination and a humoral causing antibody formation is put forward

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HUMAN RESPONSE TO FOUR TETANUS VACCINES WITH DIFFERING POTENCY WHEN ASSAYED IN ANIMALS

By

I Scheibel B L Chen M W Bentzon and K Zia

Received 10 xi 67

In order to make full use of evaluating the antigenic potency of a vaccine in laboratory animals it is necessary to have knowledge concerning the correlation between the animal and human responses

The present work deals with tetanus vaccine in this respect

Earlier examinations carried out to this end gave results which were not uniform This is not surprising since many different factors exert an influence

Ipsen (7) found the same relative values for four batches of adsorbed tetanus vaccine in animal and man and *Ikić* (4) likewise observed a positive correlation for two adsorbed vaccines *Greenberg & Benoit* (2) found positive correlation within two separate studies but when the results of the two studies are compared mutually there is lack of correlation for two of the toxoids *Holt Barnes Bousfield Spiller & Croarke* (3) conclude from their investigation that the results in guinea pigs are not misleading in direction but only in degree

Rethy Rauss Kelyi & Marocz (9) found no difference in the percentage of persons producing less than 0 05 units per ml when immunized with vaccines which showed up to three fold differences when examined in animals

Scheibel & Olesen Larsen (11) did not observe significant differences in the human response to two adsorbed vaccines with significantly different animal potency assayed in international units

We have had the opportunity of repeating such an investigation in animals and man and of extending it to four tetanus vaccines of two different types

The methods and results of the study are reported in this paper

MATERIAL AND METHODS

Vaccines

Plain tetanus toxoid No 107 partly purified by ultrafiltration 25 Lf/ml preservative 0.3 per cent phenol and 0.01 per cent thiomersalate—prepared by Taiwan Serum Vaccine Laboratory Taipei

Plain tetanus typhoid vaccine No 107-1 prepared from toxoid 107 25 Lf/ml bacterial count 0.5×10^9/ml preservative as above—prepared by Taiwan Serum Vaccine Laboratory Taipei

Alum precipitated tetanus vaccine No 20-12 the alum precipitated toxoid was washed three times with saline and resuspended to the original volume in phosphate buffered saline 25 Lf/ml alum potassium content 5 mg/ml preservative as above—prepared by Taiwan Serum Vaccine Laboratory Taipei

Purified $Al(OH)_3$ adsorbed toxoid No A-4 2000 Lf/mg N 12 Lf/ml 1 mg Al/ml preservative 0.01 per cent thiomersalate—prepared by Statens Seruminstitut Copenhagen

Potency Test in Animals

Single injections of three different doses of the four vaccines and of the international reference for plain tetanus toxoid were administered subcutaneously to guinea pigs weighing 260-280 g 15 animals were employed for each dose Blood sampling was carried out 4 weeks later and the antitoxin in each serum sample was assayed in mice by *Ipsen's* method (6)

The assays were carried out in Copenhagen in May 1962 for all the vaccines simultaneously and repeated for vaccine 20-12 in September 1962 An assay of vaccine A-4 from August 1960 is included

Field Assay

This was made during July-September 1962 on Army recruits aged 22-24 years at a training camp in Taiwan

Only those who stated that they had not been vaccinated against tetanus previously were included

The recruits were divided into four main groups to be given the four different toxoids and each group was again divided into two to receive undiluted toxoid and toxoid diluted five times with saline respectively

The injections were given deep subcutaneously under the region of the deltoid muscle When bleeding and injections were carried out on the same day the bleeding was performed first

Table 1 gives the schedule for the bleedings and injections and the doses applied

The tetanus antitoxin in each sample was assayed in mice by *Chen's* method (1) All titres are expressed in terms of the international standard tetanus antitoxin

TABLE 1

Bleeding and Injection Schedule for Human Immunization

Vaccine No	Team	Days 0			21			42			70
		Bleeding No	Inj No	ml	Bleeding No	Inj No	ml	Bleeding No	Inj No	ml	Bleeding No
107	1	P	1	0.3		2	0.3	1	3	0.3	2
107	2	P	1	1.5		2	1.5	1	3	1.5	
107-1	1	P	1	0.3		2	0.3	1	3	0.3	
107-1	2	P	1	1.5		2	1.5	1	3	1.5	2
20-12	1	P	1	0.2	1			2	2	0.2	3
20-12	2	P	1	1.0	1			2	2	1.0	3
A-4	1	P	1	0.2	1				2	0.2	3
A-4	2	P	1	1.0	1			2	-	1.0	3

P Pre immunization bleeding

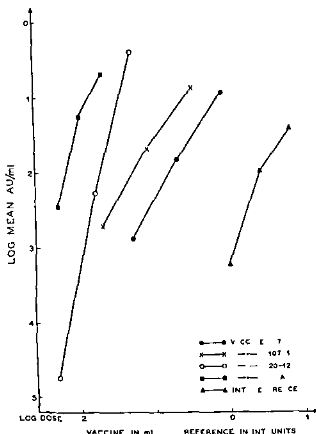


Fig 1
Dose response curves in guinea pigs for 4 tetanus vaccines and the international reference for plain tetanus toxoid May 196?

RFSULTS

Animal Experiments

The mean response in log AU per ml was calculated for each group In Figs 1 to 3 the results are presented diagramatically Table 2 gives the doses and their corresponding means and standard error the slopes and their means and standard error and the potency in inter national units

It is apparent from the diagrams and the single interval slopes in the table that the dose response relation is not described by a straight line For practical purposes the slopes of the chords have been calcu lated and used for evaluation of the potencies

It is seen that the standard error of the mean responses varies with the antitoxin level That this is caused by variations in the standard deviation is obvious from the fact that the number of animals was the same in all the groups Such variations are regularly observed with

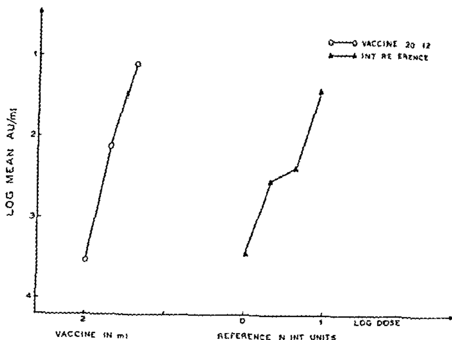


Fig 2

Dose response curves in guinea pigs for adsorbed tetanus vaccine 20-12 and the international reference for plain tetanus toxoid Sept 1962

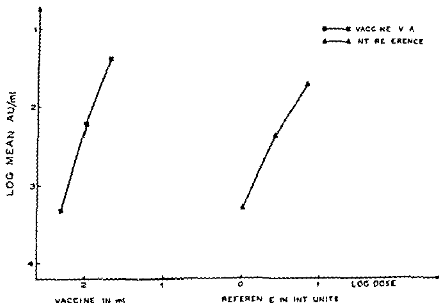


Fig 3

Dose response curves in guinea pigs for adsorbed tetanus vaccine V-A and the international reference for plain tetanus toxoid Aug 1960

TABLE 2

Log Mean Response in Guinea Pigs to Varying Doses of Four Tetanus Vaccines and the International Reference for Plain Tetanus Toxoid the Corresponding Standard Errors the Slopes and Chords and their Standard Errors

Vaccine	Dose ml	Log Mean AU/ml	SE	Slopes: Single dose intervals	Slopes: Chords	Slopes: SE	Mean slopes	SE	Potency I U /ml
107	0.05	—2.88	0.17	1.77					
May 62	0.20	—1.82	0.13	1.45	1.60	0.15			19
	0.80	—0.95	0.08				1.57	0.11	
107–1	0.02	—2.71	0.16	1.70					
May 62	0.08	—1.69	0.12	1.35	1.53	0.15			58
	0.32	—0.88	0.08						
20–12	0.005	—4.74	–	(5.0)					
May 62	0.015	—2.28	0.27	3.7	3.7	0.61			(160)
	0.05	—0.40	0.17				3.85	0.36	
20–12	0.01	—3.52	0.19	4.7					
Sept. 62	0.02	—2.11	0.16	3.4	4.0	0.40			(165)
	0.04	—1.09	0.14						
V–A	0.005	—2.45	0.15	4.0					
May 6	0.01	—1.25	0.14	1.8	2.9	0.30			(420)
	0.02	—0.70	0.11				3.10	0.23	
V–A	0.005	—3.33	0.24	3.7					
Aug 60	0.01	—2.22	0.27		3.3	0.35			(300)
	0.02	—1.38	0.13	2.8					
Tet ref plain		Int Units							
May 62	1.0	—3.21	0.20	3.1					
	2.5	—1.99	0.15	1.4	2.2	0.31			
	6.3	—1.42	0.15						
Sept 62	1.0	—3.43	0.21	3.1					
	2.0	—2.51	0.27	0.5	2.3	0.33			
	4.0	—2.35	0.25	3.2					
	8.0	—1.38	0.21				2.17	0.19	
Aug 60	1.0	—3.28	0.19	2.3					
	2.5	—2.34	0.16	1.7	2.0	0.36			
	6.3	—1.67	0.2[illegible]						

experiments of this kind and have been considered in calculating the SE of the slopes

The slopes of the chords for the two plain vaccines 107 and 107–1 vary only at random but are both significantly different from that of the international reference However it can be seen that at corresponding antitoxin levels the slopes are alike (at an antitoxin level of —1.9 to —0.88 in the May 62 assay the slopes are 1.45 1.35 and 1.40 for the two vaccines and the reference respectively) and hence at this interval potency calculation in international units is justifiable These are found to be 16 units/ml for 107 and 48 units/ml for 107 1 Calculation

TABLE 3

Potency Differences (Log) in Guinea Pigs within Two Pairs of Tetanus Vaccine of Different Types

Vaccine No			Log Difference	SE
107-1	107	(plain	0.48	0.07
V-A	20-12	(adsorbed)	0.41	0.05

based on the chords gives 19 and 58 units per ml respectively Thus the error in using the chords is only small and of no practical importance

The slopes of the chords for the two adsorbed vaccines 20-12 and V-A do not vary more than what may be due to chance but again both differ significantly from those of the international reference In the present assays this is valid also if the antitoxin level is taken into account and therefore for these vaccines the potency evaluation in international units is only approximate In future assays this complication may possibly be avoided an international reference for adsorbed tetanus toxoid having been adopted since our experiments were carried out

However in the dose response diagram given later in this paper to illustrate the relative effect of the two vaccines in animal and man it is practicable to use the international units as a dose parameter Here it is the ratio between these units which is considered and thus the above mentioned error is eliminated

For the final statistical evaluation of the animal and human relationship the relative potencies within the two pairs of vaccines have been

TABLE

Log Mean Responses in Man at Varying Stages of the Immunization with Varying and their

Vaccine No	Dose ml	No of persons	Days aft last inj	Log mean AU/ml	SE	Slope	SE
107	2 × 0.3	[illegible]	21	<−2.2[illegible]			
107	2 × 1.5	19	21	−1.63	0.19		
107-1	2 × 0.3	28	21	−1.35	0.13	0.90	0.21
107-1	2 × 1.5	23	21	−0.73	0.10		
20-12	1 × 0.2	36	21	<−2.88			
20-12	1 × 1.0	33	21	−1.96	0.17		
20-12	1 × 0.2	23	42	<−2.75			
20-12	1 × 1.0	20	42	−1.50	0.18		
V-A	1 × 0.2	36	21	<−2.47			
V-A	1 × 1.0	35	21	−1.63	0.1[illegible]		
V-A	1 × 0.2	23	42	−1.63	0.17	0.6[illegible]	0.30
V-A	1 × 1.0	30	42	−1.19	0.12		

computed This was done by standard procedures using the mean slopes for the two types of vaccines The figures are given in Table 3

As seen 107–1 was 3 0 times more effective than 107 and V–A 2 6 times more effective than 20–12 when assayed in guinea pigs

It is reassuring that the relative differences assayed in this more exact way come close to those based on the approximate potencies in international units (cf Table 2)

Field Assay

All the pre immunization serum samples contained less than 0 001 AU/ml and thus confirmed that the persons included had not been immunized against tetanus prior to this study

*The number of persons in the different groups and the mean re*sponses in log mean AU/ml at the different stages of the immunization are presented in Table 4 which also shows the standard error of the means the slopes of the dose response curves and their standard error

As can be seen the slopes show only chance variation independent of *the type of vaccine and the number of injections This permits calcu*lation of a mean value which in the present study is 0 72 This conforms well with the values which can be computed from similar earlier investigations (2 3 7 8)

As also observed by *Holt et al* (3) the dose response regression line *in man takes a significantly less steep course than in guinea pigs* particularly with the adsorbed type

The human dose response curves at the different stages of the immunization are shown in Figs 4 and 5 for the two types of vaccine

4

Doses of Four Tetanus Vaccines the Corresponding Standard Errors the Slopes Standard Errors

Dose ml	No of persons	Days aft last inj	Log mean AU/ml	SE	Slope	SF
3 × 0 3	35	18	—1 21	0 09	0 67	0 17
3 × 1 5	34	18	—0 74	0 07		
3 × 0 3	35	18	—0 82	0 08	0 90	0 16
3 × 1 5	33	18	—0 19	0 07		
2 × 0 2	36	18	—0 97	0 12	0 66	0 21
? × 1 0	3?	18	—0 51	0 08		
? × 0 ?	36	18	—0 43	0 09	0 49	0 16
2 × 1 0	36	18	—0 09	0 06		

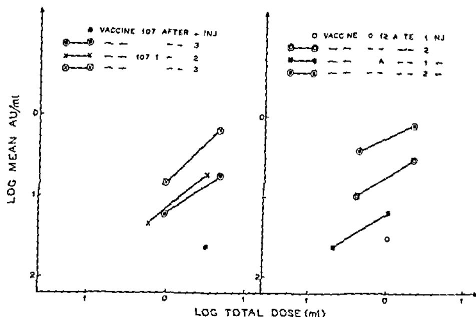


Fig 4 Dose response curves in man after 2 and 3 injections of two plain tetanus vaccines For further explanation see text

Fig 5 Dose response curves in man after 1 and 2 injections of two adsorbed tetanus vaccines For further explanation see text

Where the response to the smallest dose was not measurable in a sufficient number of persons to calculate a dependable mean (vaccine 107 and 20–12 after two and one injection respectively) only the points for the bigger doses are given

Despite the common slope calculation of the relative immunizing effect in man can only be made with any degree of certainty within each type of vaccine separately due to the differences in the number of injections and in the intervals between the injections and bleedings

The calculations are made by the standard procedures using the mean slope (0 72) and the results are presented in Table 5

Vaccine 107–1 gave a 4 5 times better result than 107 after the third

TABLE 5

Potency Differences (Log) in Man within Two Pairs of Tetanus Vaccines of Different Types

Vaccine No			Log Difference			
			after 2 inj	SE	after 3 inj	SE
107 1	107	(plain)	1 25	0 29	0 65	0 11
			after 1 inj	SE	after 2 inj	SE
A-4	20 12	(adsorbed)	>1 0	–	0 67	0 13

on 42nd day

injection and vaccine V–A is 4.7 times better than vaccine 20–12 after the second injection. As can be seen from the SE the differences are highly significant.

The results after the second injection of the two plain vaccines and after the first injection of the two adsorbed vaccines are only approximate since one of the means in each group cannot be calculated correctly due to too many titres below the measurable limit (—3.0). It is evident however that also at that stage 107–1 and V–A had a better effect than 107 and 20–12 respectively.

Persons with titres less than 0.01 AU/ml are generally considered to have only poor or no protection. The frequency and distribution of such persons during and after the immunization are shown in Table 6.

TABLE 6

Frequency of Persons with less than 0.01 Units/ml at Varying Stages of the Immunization with Four Different Tetanus Vaccines

Vaccine No	Dose ml	Days after last inj	<0.01 AU/ml	Dose ml	Days after last inj	<0.01 AU/ml
107	2 × 0.3	21	14/22 = 63.5%	3 × 0.3	18	2/35 = 5.7%
107–1	2 × 0.3	21	5/28 = 18.0%	3 × 0.3	18	1/35 = 2.9%
107	2 × 1.5	21	6/19 = 31.4%	3 × 1.5	18	0/34
107–1	2 × 1.5	21	1/23 = 4.4%	3 × 1.5	18	0/33
20–12	1 × 0.2	21	34/36 = 94.5%			
V–A	1 × 0.2	21	29/36 = 80.1%			
20–12	1 × 0.2	42	21/23 = 91.6%	2 × 0.2	18	2/36 = 5.6%
V–A	1 × 0.2	42	6/23 = 26.1%	2 × 0.2	18	1/36 = 2.8%
20–12	1 × 1.0	21	15/33 = 45.3%			
V–A	1 × 1.0	21	9/35 = 25.8%			
20–12	1 × 1.0	42	5/20 = 25.0%	2 × 1.0	18	0/32
V–A	1 × 1.0	42	2/30 = 6.7%	2 × 1.0	18	0/36

At all stages vaccination with 107–1 resulted in lower percentages of poorly protected persons than vaccination with 107. The same applies after vaccination with V–A as compared with 20–12. Not all differences are significant but the trend is quite convincing.

Relationship of Human and Animal Effectiveness

In Table 7 the relative potencies within the two pairs of vaccine in animals and man are presented together. For man only those after the third injection of the plain vaccines and after the second injections of the adsorbed vaccines are given.

With both types there is positive correlation between the relative potencies in animal and man. The vaccines giving the highest response in animals also give the highest response in man. The potency differ

TABLE 7

Potency Differences (Log) in Guinea Pigs and Man within Two Pairs of Tetanus Vaccines of Different Types

Vaccine No		Guinea Pigs		Man	
		Log Difference	SE	Log Difference	SE
107–1	107 (plain)	0.48	0.07	0.65	0.11
V A	70–12 (adsorbed)	0.41	0.05	0.67	0.13

ences are numerically more pronounced in man than in animals but from the statistical viewpoint this may be incidental

The response in man in relation to the animal potency expressed in I U is illustrated by the right hand curves in Figs 6 and 7

Fig 6 represents the plain type after the third injection and Fig 7 the adsorbed type after the second injection the results at the earlier stages of the immunization being too incomplete to utilize

The vertical lines indicate the ranges of variation for the means

The irregularities of the curves may as seen be due to chance The slopes can be estimated to be about 0 85 for both types this conforms well with the mean slope 0 72 found when the volume is used as the dose parameter The slight difference reflects the above mentioned tendency towards higher relative potency in man than in animal The

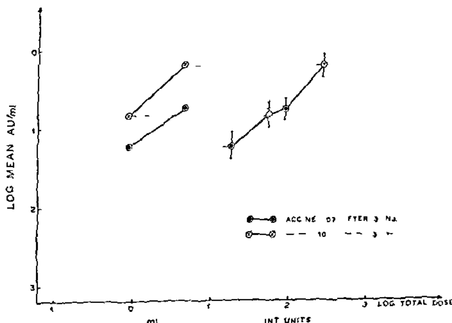


Fig 6

Relationship between the average response in man and the doses of plain tetanus vaccine expressed in I U For further explanation see text

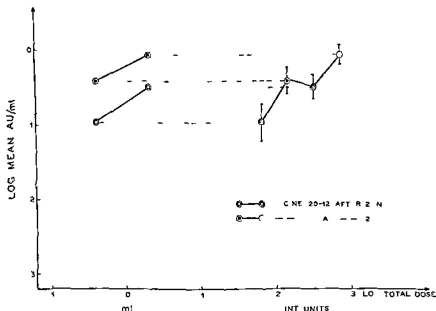


Fig 7

Relationship between the average response in man and the doses of adsorbed tetanus vaccine expressed in I U For further explanation see text

dose response regression lines from Figs 4 and 5 have been included on the left side of Figs 6 and 7 respectively to illustrate the relationship between the two dose parameters It is obvious that the animal potency expressed in I U is a better yardstick than the volume or Lf (cf p 2) for the effect in man of different batches of vaccines of the same type applied according to the same schedule

DISCUSSION

In a previous investigation (11) no significantly positive correlation could be demonstrated between the relative potencies in man and guinea pigs of two adsorbed tetanus vaccines The trial in man was carried out with only one dose level and therefore gave no information as to the slope of the human dose response regression line

In the present field trial two dose levels were used The slopes have been calculated where possible and have been found to vary at random around a mean value of 0 72 independent of the type of vaccine and the stage of the immunization

Assuming that this is a generally acceptable slope under our conditions we have used it to test whether the human responses observed at the first trial were compatible with the potency difference in question

It appeared that the slightly higher response to the vaccine with the highest potency found two weeks after the second injection (difference

0.04, SE 0.10) did not vary significantly from the calculated value (difference 0.30, SE 0.11) whereas the lower response to this vaccine four weeks after the first injection was not compatible with the expected result. The reason for this is not known but may be sought in the less exact assaying of the means at the low levels of antitoxin obtained at that stage.

The results of the present investigation and the possibility that the lack of agreement between our two trials at a well defined stage of the immunization may have been incidental, together with the positive correlation observed by other investigators (2, 3, 4), in our opinion justify the acceptance of a positive correlation between the relative effect in man and animal as a fairly well established fact.

We have not been able to confirm the observation by *Ikic* (5) that the slopes of the dose response curve in man and guinea pigs are alike. Our results on this point are in accordance with those obtained by *Holt et al.* (3) who found a significantly flatter course in man than in guinea pigs. The fact that the slope varies with the antitoxin level and that the same applies to the standard deviation makes it understandable that many different values are mentioned in the literature, and makes comparison between these rather complicated and often illusory.

The background for investigating the relationship between the responses in man and animal is the possibility of being able to predict the effect in man from the animal potency.

Due to the different slopes in man and animal the response to dose variations in man cannot be estimated from animal assays as was pointed out by *Holt et al.* already in 1959 (3).

The positive correlation between the relative potencies in man and animals which has been observed by several investigators and under different conditions, on the other hand, implies the possibility of expressing a human dose requirement in terms of the animal potency.

Before this is feasible there are, in our opinion, four claims which must be fulfilled.

1) *The ratio of the relative potency in man and animal must be 1.*

If this is not so, it must mean that the vaccines differ in quality and that the ratio must be determined for each batch of vaccine, which is impracticable. The present study indicates that the ratio may be one, but as there is no clear cut evidence in support of this to be found in the literature, it needs further investigation.

2) *The mean antitoxin response in man to be aimed at at a given stage of the immunization schedule must be settled.*

The choice of this must rest with the vaccine and schedule used in each country.

Where adsorbed vaccines are concerned we would suggest 2 weeks

after the second injection as a convenient stage This will most likely be a more sensitive period than after the final injection at an earlier stage too many persons may not have responded with measurable amounts of antitoxin

Concerning the level of antitoxin we know from field trials in Denmark that under our conditions a mean of about 0 3 AU per ml 2 weeks after the second injection guarantees a mean of about 10 units after full vaccination (12)

In another field trial carried out with a similar vaccine but where the maximum response was not measured we found that 95 per cent were still well protected 12 years after their last injection (10) Although there is a missing link between these two experiences we would think 0 3 AU a safe level but more knowledge is required to settle this

3) *The dose response regression line in man of a vaccine with known potency in I U must be at hand using the same immunization model as agreed for point 2*

This is necessary in order to ascertain the numbers of international units required to obtain the desired antitoxin level and to estimate the range in response variation which may result from the inaccuracy of the potency assay

In the present study on adults in Taipei the dose response curve shows that the antitoxin level suggested by us was obtained with a total of about 200 I U of the adsorbed vaccine 18 days after the second injection nearly the same has been observed for adults in Denmark (11)

In Yugoslavia 0 7 AU per ml after a total of about 170 I U was observed 2 weeks after the second injection (5)

The slope in man found in the present study and the limit of error of our potency determination (50–200 per cent) would imply a response variation of ± 0 24 in log titres

Whether the position of the curve and the slope given here can be accepted as generally valid needs further documentation

4) *The potency in I U of the vaccines must be known*

With the establishment of an international standard also for adsorbed tetanus vaccine this should no longer involve overwhelming problems Ways of decreasing the limits of error of the method would be valuable

Once the three first points are settled the potency assay is the only procedure which must be carried out for each batch of vaccine

It seems however that although a considerable amount of knowledge on each of the four points has accumulated during the years there

is still a long way to go before the animal potency can be considered as a well defined parameter for the effect in man

SUMMARY

The relative potencies in guinea pigs and man of two pairs of plain and two pairs of adsorbed tetanus vaccines have been investigated and compared

A positive correlation was observed The ratio between the relative potencies in animals and man did not deviate significantly from one though there was a tendency towards higher relative potency differences in man than in animals

The slope of the dose response regression line for the plain type of vaccine was significantly less steep than that of the adsorbed type when assayed in guinea pigs In man the slopes were found to be alike for both types and less steep than the slope for the plain vaccine in animals The human slope was independent of the stage of immunization

The conditions for expressing the dose requirement for man in terms of the animal potency are discussed

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COMPARATIVE TESTING OF PRECIPITATION METHODS FOR QUANTITATION OF C REACTIVE PROTEIN IN BLOOD SERUM

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The determination of C reactive protein (CRP) in blood serum has proved to be of diagnostic value in relation to various diseases (13 19 38) The most commonly used method for the demonstration of CRP is the capillary tube precipitation test (CT test) (2) but immunodiffusion techniques have also been applied for this purpose (4 5 10 11 18 21 24 35 44) A greater sensitivity of the double diffusion in gel method (DD method) when performed in a micromodification was noted in an earlier investigation (30) Neither technique however is sufficiently reliable from a quantitative point of view

Quantitative techniques have also been applied to the determination of CRP A spectrophotometric technique based on C polysaccharide precipitation of CRP from serum was described by *Wood & McCarty* (43) *Libretti Kaplan & Goldin* (22) used a modified Oudin technique Another agar diffusion technique was devised by *Fukuda Heiskell & Carpenter* (9) utilizing the relative position of the precipitation line between the antigen and antibody basins for quantitation *Ray & Shay* (36) recently described a precipitin—inhibition technique

The single radial immunodiffusion (halo) technique as described by *Mancini Carbonara & Heremans* (25) is a simple sensitive and accurate immunochemical technique for quantitation of *e g* blood serum proteins The aim of the present investigation was to evaluate this halo technique for CRP determination and to examine the influence of some factors which might limit its applicability Furthermore the CRP concentrations found in blood sera with this technique have been compared with the results obtained by means of the CT technique and comparative DD analysis

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MATERIAL AND METHODS

Antigens

Blood sera Group A During 1963 a combined health examination and population study of 973 randomly selected men all of whom were born in 1913 was performed at the Sahlgren Hospital in Goteborg (41) Eight hundred and fifty five subjects (88 per cent) were examined in the hospital The non participating group was analysed separately (40) Blood sera from 835 of the examined individuals were available and comprise group A in the present investigation The decanted sera were frozen in portions within 24 hours after venipuncture and kept at —20 C until the analyses were performed

Group B One hundred and thirty five samples selected from sera sent to the Bacteriological Laboratory of the Sahlgren Hospital Goteborg for CRP determination were analysed Only sera found CRI positive with the CT technique were included in this series

Reference antigen A CRP containing pleural fluid was used as a reference antigen in the comparative DD analyses as well as in the halo technique The CRP concentration of this reference antigen was estimated at 85 μg CRP/ml with the halo technique by comparison with a purified CRP preparation (see below)

Antisera

CRPA used for the halo technique was produced in a goat by injection of purified human CRP as described in an earlier paper (28) For the CT and DD techniques commercially available CRPA (Hyland Lab Calif USA) was employed In some of the diffusion coefficient analyses of CRP a rabbit anti human CRP (Hyland Lab) was used The antibody content of the antisera employed has been described elsewhere (28)

Anti human serum A polyvalent antiserum produced in sheep against normal serum proteins was employed

Quantitation of CRP in Blood Serum

The capillary tube precipitation test (CT test) for determination of CRP ( ) was performed with the CRPA undiluted and diluted 1 4 Equal amounts of antiserum and blood serum were thoroughly mixed in the capillary tube After two h urs incubation at 37 C the tubes were transferred to the refrigerator Results were recorded after 24 and 48 hours incubation at 4 C The results obtained with the CT technique were expressed as the height of the precipitate in mm Precipitates less than 1 mm were recorded as traces The results with the group A sera prese ted in this study are those registered after 48 hours incul ation since in this in t nce

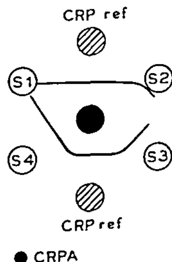


Fig 1
Comparative double diffusion in gel analysi f r demonstration of C reactive protein in four [illegible] sera (S 1 – S 4) by means of CRPA (center [illegible]) and a CRP reference antigen (upper and l er hatched ba ins) Sera reacting like S 1 were cla i fied — S 2 (+) S 3 ++ and S 4 +++ Se a giving precipitation lines intermediate in p siti n between those illustrated by S 2 and S 3 were classifie l +

TABLE 1

Statistical Analysis of Results obtained with the Halo Technique

CRP concentration in per cent of reference	Area of precipitation ring (in units) Plate 1			Plate 2			Plate 3			Mean	S D	Coefficient of variation
100	73.9	75.5	73.9	83.2	78.5	77.0	77.0	77.0	77.0	77.0	2.79	3.6
75	62.1	56.8	60.8	66.5	63.6	56.8	62.1	60.8	59.5	61.0	3.10	5.1
50	41.9	45.4	44.2	44.2	46.6	41.9	47.8	45.4	46.6	44.9	2.05	4.6
5	27.4	27.4	27.4	27.4	29.[illegible]	28.3	28.3	30.2	28.3	28.2	0.97	3.4
10	19.6	19.6	18.9	18.1	18.1	18.1	18.9	18.9	18.1	18.7	0.63	3.4
Equation of regression line	$\hat{y}x = 0.621x + 12.7$			$\hat{y}x = 0.683x + 11.0$			$\hat{y}_x = 0.645x + 12.9$					

Equation of common regression line $\hat{y}x = 0.65x + 12.2$ $r = 0.995$ $S_{y.x} = 2.10$

SD = *standard deviation* r = *regression coefficient* $S_{y.x}$ = residual standard deviation

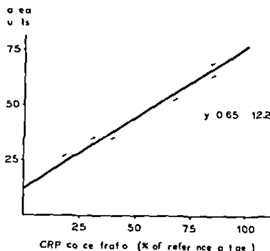


Fig 2
Graphic representation of the common regression line and its 95 per cent confidence limits calculated from values obtained with the halo technique (cf Table 1)

the intent was to make this technique as sensitive as possible These reactions exceeded or at least equalled those observed after 24 hours incubation The results with the sera belonging to group B on the other hand were those recorded after 24 hours incubation

The double diffusion in gel method (DD method) of *Ouchterlony* (33) was employed in the micromodification of *Wadsworth* (49) A seven basin arrangement was used see Fig 1 The antiserum (CRPA) was in the central basin the CRP containing reference antigen in two opposed peripheral basins and blood sera in the remaining four basins This permitted the formation of two parallel reference precipitation lines whose deviation was registered A rough semi quantitative estimation was made depending on the position of the precipitation line in relation to the blood serum and antibody basins (see Fig 1)

The single radial immunodiffusion (halo) technique as described by *Mancini et al* (25) was employed slightly modified The goat anti human CRP diluted 1/25 with physiological saline was mixed with an equal volume of 3 per cent agar (Difco Noble agar) in 0 85 per cent NaCl solution To reduce nonspecific precipitation around the wells 1 part Pentosanpolysulphoester (SP 54 Benechemie München Solln West Germany) was added to 99 parts CRPA agar (11) The CRPA agar was poured between two 8 × 8 cm glass plates separated by a 1 mm thick steel frame After gelification and removal of the upper glass plate and the frame 16 circular holes 2 mm in diameter were punched out and filled with 2 µl (Carlsberg pipettes AB Grave Stockholm Sweden) of the serum to be tested or the reference CRP antigen Six different dilutions of the reference antigen the CRP concentrations of which were calculated to cover the range of the CRP concentrations in the samples were tested on each plate After 14 days of incubation at room temperature in a humid atmosphere the plates were eluted for three days with several changes of saline dried and stained with amidoblack Perpendicular diameters of the precipitation rings were measured to the nearest 0 06 mm with a moveable stage microscope and the area of the ring plus basin was calculated on the basis of the average diameter CRP concentrations in the sera were determined from the regression line (area versus concentration) plotted for the dilutions of the reference antigen Sample with CRP concentrations exceeding that of the reference antigen were estimated from the extrapolated regression line Control determinations were performed on proper dilutions of some of these sera and the values showed good agreement with the extrapolated values obtained with the undiluted sera Sera giving no precipitation rings have been recorded as CRP positive or negative according to the results of the DD analyses. With the halo technique concentrations as low as 1 per cent of the CRP concentration of the reference antigen corresponding to about 1 µg CRP/ml could be quantified This corresponds well with the sensitivity of the technique as reported by *Mancini et al* (25)

Reproducibility of the halo technique was examined in the following manner Saline dilutions of the standard reference CRP antigen were prepared to contain 75

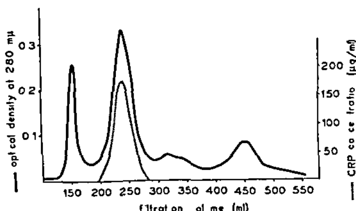


Fig 3

Gel filtration of crude CRP as obtained after chromatography on DEAE cellulose The separation was performed on a 2 5 × 85 cm column of Sephadex G-200 equilibrated with 0 05 M Tris HCl buffer pH 7 3 Flow rate 12 ml/h temperature +6 C

50 25 and 10 per cent of the initial CRP concentration The reference antigen and the dilutions were each tested in triplicate in each one of three immune serum agar plates The positions of the different dilutions were randomly selected and varied in the three plates The values of the areas obtained are given in Table 1 in which is included the equation of the regression line representing each plate calculated by standard statistical methods The linearity and parallelism of the regression lines tested by means of analyses of covariance did not differ significantly at the 99 per cent significance level A graphic representation of the common regression line and its 95 per cent confidence limits comprise Fig 2 The coefficient of variation (standard deviation in per cent of mean value) obtained for each dilution is given in Table 1

Preparation of Purified CRP Used as Standard

Human CRP was isolated from serous fluids with a modification of the method *described by Hokama & Riley (17)* and *was used as the standard for determining the* CRP concentration in the reference antigen A sample of 800 ml of CRP-containing pleural fluid was dialysed at 4 C against several changes of a 0 05 M sodium citrate buffer pH 7 0 containing 0 1 M NaCl The dialysed material was chromatographed on a 4 × 23 cm column of DEAE cellulose (Serva Entwicklungslabor Heidelberg West Germany) which had been equilibrated with the same citrate buffer and packed at a pressure of 10 p s i according to the suggestion of *Sober et al* (39) Under these conditions the CRP was retained b the DEAE cellulose and the material eluted d d not contain any demonstrable CRP The column was thoroughly washed with the same buffer The retained material was eluted by application of the aforementioned citrate buffer but with the salt concentration increased to 1 5 M NaCl The CRP containing material was eluted *between 200 and 350 ml* and *appeared as a single* slightly assymetric peak

After concentration by ultrafiltration the eluted CRP containing material was further purified by gel filtration (8) Samples of 5 ml were passed through a 2 5 × 8 cm column of Sephadex G 200 (AB Pharmacia Uppsala Sweden) by elution with 0 05 M Tris HCl buffer pH 7 3 Fractions of 5 ml were collected Three major peaks appeared as may be seen in the elution diagram of Fig 3 CRP was symmetrically localized in the second peak The CRP concentration as dete mined by the halo tech nique sho ved good agreement with the absorption curve at 280 mμ The CRP con taining fractions from different gel filtration experiments were pooled and concen trated by means of ultrafiltration The concentrated material was refiltered on a 2 5 × 85 cm column of Sephadex G-200 equilib ated and eluted wi h 0 05 M Tris

HCl buffer pH 7.3 or 0.001 M phosphate buffer pH 7.0 containing 0.1 M NaCl. The elution diagram of this second gel filtration showed only small amounts of UV absorbing (280 mμ) CRP negative matter. Polyacrylamide electrophoresis (6.3%) performed on the concentrated CRP containing material revealed one distinct band.

To test the purity of the preparation antisera were produced in rabbits. The material was emulsified in Freund's complete adjuvant and injected subcutaneously into two rabbits three times at one week intervals. During the next 6 months 4 booster doses were given and the rabbits were bled 10 days *after the last injection*. In total 0.4 mg of antigen was given to each of the rabbits. The antisera obtained gave only one precipitation line against human CRP containing serum or pleural fluid when tested with the DD technique. By means of comparative DD analysis *this precipitate was shown to correspond to a CRP–CRPA reaction*.

The protein concentration of the purified CRP preparation was determined according to the method of *Lowry et al* (23) with crystalline lysozyme (Nutritional Biochemicals Corporation, Cleveland, Ohio, USA) as protein standard. The figure thus *obtained* was fairly close to that obtained by calculating data from amino acid analyses of the CRP preparation (29).

Gel Filtration of CRP Positive Sera

Five ml portions of ten different blood sera containing high concentrations of CRP were gel filtered through a column of Sephadex G-200 (2.5 × 85 cm) which *had been equilibrated with 0.05 M Tris HCl buffer pH 7.3. The absorbance was* measured at 280 mμ in a Beckman DU spectrophotometer. Localization of the CRP in the elution diagram was performed by testing the eluted 5 ml portions with the comparative DD technique as previously described (30). Quantitation of CRP was performed with the modified halo technique described above.

Determination of Diffusion Coefficients

The diffusion coefficient of CRP was determined in 50 sera selected from group B to contain different CRP concentrations. The method described by *Allison & Humphrey* (1) using two basins (2 × 18 mm) at right angles was used. With this arrangement the ratio of the diffusion constants of antigen (D_g) and of antibody (D_b) is given by $tg\ \Theta = \left(\frac{D_g}{D_b}\right)^{1}$ where Θ is the angle between the precipitation line and the antigen basin. The CRPA concentration was preliminarily adjusted *according to the CRP concentration of the serum determined as described above*. In all of these analyses the goat antiserum was used. Five additional sera were tested with the aforementioned antiserum as well as with a rabbit anti human CRP immune serum. The ultimate criterion of balance was the formation of a sharp and *distinct precipitation line which did not move during the diffusion period*. The angle Θ was determined from a projection of the photographic negative taken after 72 hours of diffusion.

RESULTS

The incidence of C-reactive protein in the sera belonging to group A as shown by *the CT technique with the undiluted CRPA was compared* with that found by the DD technique (see Table 2). By means of the CT technique 30 of the 835 investigated sera (3.6 per cent) were found to be CRP positive (precipitate ≥ *1 mm*). *29 of these were positive and* one was negative when tested with the DD technique. This latter serum was obtained from a man who upon clinical or laboratory examination did not show any apparent abnormalities except for a high serum concentration of triglycerides (2.57 mmol/ml). Of the 178 sera (21.3 per cent) showing trace amounts of CRP with the CT technique 172 were positive with the DD technique and 6 negative. Finally 627 of the sera

(75.1 per cent) were negative with the CT technique. In 500 of these sera CRP was demonstrated with the DD technique. CRP was thus demonstrated in 701 (84.0 per cent) of the group A sera with the DD technique.

When the CT technique was modified so that the commercial CRPA was used diluted 1/4 the following results were obtained (Table 3). Of the 835 sera tested 41 (4.9 per cent) were positive (precipitate $\geq$ 1 mm) by the CT technique; all of these were also positive with the DD technique. Of the 307 sera (36.8 per cent) showing trace amounts of CRP as demonstrated with the CT technique 297 were positive with the DD technique whereas ten were negative. Finally 487 sera (58.3 per cent) were negative by the CT technique but in 363 of these CRP could be demonstrated by the DD technique.

TABLE 2

Comparison of the CRP Results Obtained with the Capillary Tube Precipitation Test and the Double Diffusion in Gel Technique (Group A)
CRPA Undiluted in the CT Test

	Precipitate in mm	Double diffusion in gel: Negative	(+)	+	++	+++	Total
Capillary tube precipitation test	9	1				1	2
	4				1		1
	3				3	2	5
	2		1		5	4	10
	1			1	11		12
	Traces	6	95	49	27	1	178
	Negative	127	342	123	34	1	627
	Total	134	438	173	81	9	835

TABLE 3

Comparison of the CRP Results Obtained with the Capillary Tube Precipitation Test and the Double Diffusion in Gel Technique (Group A)
CRPA Diluted 1:4 in the CT Test

	Precipitate in mm	Double diffusion in gel: Negative	(+)	+	++	+++	Total
Capillary tube precipitation test	3					2	2
	2		1		8	4	13
	1		1	4	19	2	26
	Traces	10	147	103	46	1	307
	Negative	124	289	66	8		487
	Total	134	438	173	81	9	835

TABLE 4

Comparison of the CRP Results Obtained with the Capillary Tube Precipitation Test Using CRPA Undiluted and Diluted 1:4 (Group A)

	Precipitate in mm	CRPA diluted 1:4 Negative	Traces	1	2	3	Total
CRPA undiluted	9			1		1	2
	4				1		1
	3			1	3	1	5
	2			2	8		10
	1		3	8	1		12
	Traces	20	149	9			178
	Negative	467	155	5			627
	Total	487	307	26	13	2	835

The results obtained with the CT technique using commercial CRPA undiluted compared to those obtained when the CRPA was diluted 1/4 are summarized in Table 4. The diluted CRPA revealed CRP in 160 (25.5 per cent) of the 627 sera which were negative when tested with undiluted CRPA. On the other hand, of the 208 CRP containing sera including trace reactions as detected with undiluted CRPA, 32 showed a weaker reaction (20 negative) when tested with diluted CRPA, whereas ten showed a stronger reaction. Nine of these latter sera belonged to the group showing trace amounts with undiluted CRPA.

The quantitative distribution of 825 group A sera correlated to the reactions obtained with the CT technique using undiluted CRPA may be seen in Table 5. Sera without demonstrable CRP as determined by the CT technique ranged from 0 to 12 μg CRP/ml, while sera showing trace amounts ranged from 0 to 25 μg CRP/ml. Also in the sera with higher precipitates as demonstrated with the CT technique a variation of the CRP concentration as measured with the halo technique was obvious when the sera were grouped according to the results obtained with the CT technique. This condition is still more evident from Fig. 4, in which the quantitative distribution of 135 CRP containing sera from patients with various clinical conditions (group B) is correlated to the results obtained by the CT technique.

Twenty group A sera giving weak positive ((+)) reactions when tested with the DD technique were retested after inactivation of the sera as well as the CRPA by heating to 56 C for 30 minutes and they still gave a weak deviation (positive reaction) of the reference precipitation line. This demonstrates the complement independence of the precipitating system.

A representative elution diagram obtained after gel filtration of a CRP containing serum is seen in Fig. 5. The CRP appeared between the second and third peaks.

TABLE 5

Comparison of the CRP Results Obtained with the Capillary Tube Precipitation Test and Immunodiffusion in 825 Sera (Group 4) CRPA Undiluted in the CT Test CRP Concentrations Determined with the Halo Technique Sera Labelled Negative Had no Demonstrable CRP with DD Analysis

	Precipitate in mm	Immunodiffusion CRP concentration in μg/ml: Negative	<1	1–4.2	4.3–8.4	8.5–12.7	12.8–25.4	25.5–42.4	42.5–85	>85	Total
…lary	9	1								1	2
	4						1				1
…pitation	3						1	3	1		5
	2		1			1	4	3	1		10
	1			[illegible]	[illegible]	4	5	1			12
	Traces	6	95	36	20	13	7				177
	Negative	127	332	106	43	10					618
	Total	134	428	142	65	28	18	7	2	1	825

…n sera unavailable for quantitation with halo technique

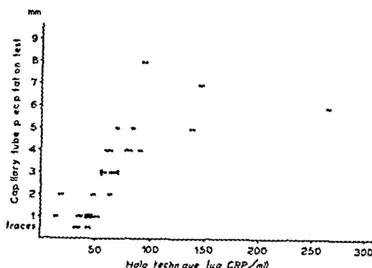


Fig 4

Relation between CRP concentration determined by the halo technique and by the capillary tube precipitation test in 135 CRP-containing sera (group B)

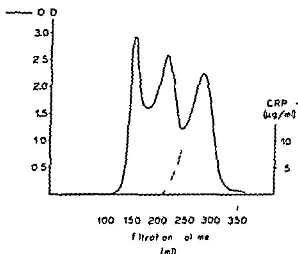


Fig 5

Elution pattern of a CRP containing human serum separated on Sephadex G 200
Experimental conditions were the same as described in Fig 3

The mean and standard deviation of the angle Θ determined for the CRP in 50 sera from patients with different clinical conditions was 41.0 ± 0.7 (range 39.7 — 42.2) The corresponding value for the purified CRP preparation used for calibration of the quantitative method was 40.8 When five sera were tested with both the goat and the rabbit antiserum the angles obtained did not differ from the mean calculated from the fifty tests by more than one standard deviation

DISCUSSION

Though occurring nonspecifically the presence of CRP in blood serum has been shown to be of diagnostic as well as prognostic value in various clinical conditions for a review see ref 38 However for quantitation of CPP in blood sera the routinely performed CT technique has certain disadvantages This has been pointed out by *Nakamura et al* (27) who described varying reactivities of commercial CRPA anti sera even if they were obtained from the same manufacturer The need for a standardisation of CRP determinations is also illustrated by the results compiled in Table 5 and Fig 4 from which it may be seen that sera giving the same result when tested for CRP with the CT technique show a considerable range in CRP concentration as determined by the halo technique In the tests performed with the group B sera the same batch of CRPA was employed

As was also shown in an earlier investigation (30) the presence of CRP as demonstrated with the CT technique in most instances could be verified by the comparative DD technique However in 7 group A sera reacting positive in the CT technique the presence of CRP could not be verified by the DD technique (Table 2) Such false positive reactions have been ascribed to precipitated lipoproteins (7) *Knights et al* (20) report that originally negatively reacting serums may become positive under several varieties of conditions such as after storage either frozen or at +4 C These reactions were often correlated to bacterial contamination but were also shown to occur in sterile sera When retesting negative sera after several weeks of storage using the DD technique the presence of CRP has never been noted (unpublished observations) which indicates that these precipitates obtained with the CT technique were non specific and not due to the presence of CRP

About 80 per cent of the sera which were negative with the CT technique were shown to contain CRP by means of the DD technique (cf Table 2) This is a much higher frequency than reported in an earlier study (30) and the cause is probably to be found in the basin arrangement used In the present study a seven basin arrangement was used with a reference antigen in two opposed peripheral basins Only a very low CRP concentration ($\approx$ 0 5 μg CRP/ml) is needed to cause a slight deviation of the CRP CRPA reference line whereas this low amount is not sufficient to form an independent precipitation line

Comparison of the results obtained when CRPA was used undiluted and diluted 1/4 in the CT technique showed that the diluted CRPA gave some 17 per cent more positive reactions than the undiluted CRPA (Table 4) About 20 per cent of the total number of the sera showed a higher value when the diluted CRPA was employed whereas in 4 per cent a lower value was recorded These differences probably reflect the importance of approximating optimal proportions when the CT technique is performed This is in conformity with the conclusions arrived

al by *Nakamura et al* (27) The DD technique forms precipitates with in a wide range of concentration of the reactants

Since neither the CT nor the DD technique as performed in this study can be regarded as more than semi quantitative a more precise technique for quantitation of CRP was tested namely the halo technique as described by *Mancini et al* (25) The quantitative technique of *Wood & McCarty* (43) is sensitive but requires pneumococcal C polysaccharide which is rather laborious to prepare With a modified Oudin technique *Libretti et al* (22) measured as low concentrations as 3 μg CRP/ml The sensitivities of the agar diffusion techniques devised by *Fukuda et al* (9) and *Crockson* (5) are hard to evaluate since no absolute values are given The precipitin inhibition technique described by *Ray & Shay* (36) detected between 8 0 and 16 5 μg CRP/ml serum depending on the accuracy of the serological dilutions The halo technique was considered as a simple and suitable method which allows quantitation of concentrations of about 1 μg CRP/ml It is thus about ten times as sensitive as the precipitin inhibition technique described by *Ray & Shay* (36) Furthermore this technique seems more suitable and accurate since titration is not required The coefficient of variation obtained in the present investigation varied between 3 4 and 5 1 per cent (Table 1)

Hokama, Coleman & Riley (14) however found different diffusion coefficients for CRP when analysing sera with the angle plate technique of *Allison & Humphrey* (1) This should mean that quantitative agar diffusion techniques would be unsuitable for the determination of the CRP content of different sera Differing results were also obtained when various buffers were used for the solution of the agar It was therefore considered necessary to analyse diffusion under the experimental conditions used with the halo technique The balance of the precipitating CRP CRPA system was carefully controlled by preliminary concentration determinations of the CRP containing sera and a relatively long diffusion time The angle Θ varied between 39 7 and 42 2 (mean = 41 0 ± 0 7) Although *Allison & Humphrey* reported that the values of Θ should lie within 1° the variation obtained in this study of sera with widely differing CRP concentrations is probably within experimental error The mean value of the angle Θ is close to that reported by *Hokama et al* (14) for their agar saline system but the standard deviation in the present series is 0 7 as compared to 4 06 (range 32 7 – 48 42) in their series The difference is difficult to explain but might depend on different batches of agar or possibly imbalance of the systems tested The average molecular weight of CRP has been estimated at 118 000 with components ranging from 110 000 to 144 000 (12) The elution pattern of CRP at gel filtration of CRP containing sera on Sephadex G 200 is in accord with these values (cf Fig 5) The molecular weight of rabbit IgG has been determined at 137 000 145 000 (3 26 34) Since the testing of the same CRP sera

with the technique of Allison & Humphrey gave approximately the same value of Θ when either a rabbit or a goat antiserum was used it can be supposed that the diffusion coefficients and molecular weights of rabbit and goat IgG are approximately the same If this is so one would have expected the angle Θ to be slightly larger or close to 45 Since it is not this is consistent with the indicated interaction between CRP and the agar as suggested by *Hokama et al* (14) In the present study however this possible interaction does not seem to vary for CRP in different sera and thus the objections of *Hokama et al* (14) against agar diffusion techniques for quantitation of CRP do not seem to be valid under the experimental conditions used

Another important factor is the quality of the CRP used for standard ization of the halo technique Purification by means of chromato graphy on DEAE cellulose with subsequent gel filtration on Sephadex G 200 seems to provide a sufficiently pure material which neither reacts with polyvalent antiserum to normal serum proteins nor in duces the formation of antibodies other than anti CRP when injected into rabbits The diffusion conditions of the purified CRP as deter mined in the present study are close enough to those of native CRP to justify its use as a standard reference preparation Recent investiga tions by *Hokama Coleman & Riley* (15 16) have indicated that in native sera the CRP occurs in equilibrium with a mucopolysaccharide which elutes in the first peak (cf Fig 3) at gel filtration of CRP iso lated by chromatography on DEAE cellulose The nature of this rela tionship and especially its influence on the diffusibility in agar of native CRP from different sources and under various diffusion con ditions remains to be more clearly established

In Table 5 and Fig 4 the distribution of the quantitative CRP values in groups A and B is correlated to the results obtained with the CT technique Although a relation is obvious the distribution of the quan titative values is very wide within each CT level Only with traces and 9 mm of precipitate was the maximum concentration less than twice the minimum concentration and at some levels the maximum concen tration exceeded the minimum concentration by more than five times

It seems reasonable to assume that a precise quantitation technique for the estimation of CRP in blood serum would be advantageous when the development of the CRP response is to be correlated to the course of a disease The halo technique seems to be well suited for this pur pose Not only is the technique precise and sensitive but the amount of antiserum required is very low Thus 0 01 ml of the goat antiserum was sufficient for the quantitation of CRP in at least 10 sera In the present study the diffusion plates have been incubated for two weeks but a diffusion time of 1–3 days would have been sufficient By in creasing the antiserum concentration the diffusion time necessary to obtain balance can be diminished but only with some loss of sensitivity The plates may be read without elution and staining and thus provided

proper reference antigens are used preliminary results are obtained as quickly as with the CT technique One further advantage is the immunological specificity of the technique

Finally it should be stated that the frequent occurrence (84 per cent) of CRP in the sera of randomly selected men (group A) is unexpectedly high since most of the men were in active work (41) *Some of the clinical implications* of the results obtained with these more sensitive immunodiffusion techniques will be presented elsewhere (31 37)

SUMMARY

CPR has been determined in sera from 835 randomly selected middle aged men by means of diffusion in gel techniques and precipitation in capillary tubes With the diffusion in gel technique 84 per cent of the sera were shown to contain CRP whereas CRP could be demonstrated in only 25 per cent by the capillary tube precipitation test The results *obtained with the latter technique were compared with the CRP con*centrations as determined by the single radial immunodiffusion (halo) technique It was concluded that the capillary tube precipitation test is inferior to immunodiffusion methods regarding sensitivity preci*sion and specificity*

For standardisation of the quantitative halo technique a pure CRP preparation is necessary A simple modified procedure for obtaining a CRP preparation which fulfills immunologic criteria of purity is described

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THE HISTOLOGY OF THE GASTRIC MUCOSA IN PERNICIOUS ANAEMIA

By

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Received 7 ix 67

The gastric mucosa in patients with pernicious anaemia (p a) invariably shows pathological changes Although it is generally agreed that atrophy of the specific glands are present it has been much discussed to what degrees atrophy cellular infiltration and intestinal metaplasia are present

The atrophy of the gastric mucosa in a patient with p a was first recorded nearly 100 years ago (*Fenwick* 1870) The famous study by *Faber & Bloch* (1900) who shortly after death of p a patients fixed their stomachs in situ showed that atrophy and pronounced cellular infiltration invariably were present in this condition They named it gastritis interstitialis progressiva atroficans

In his monography from 1935 *Faber* stressed the importance of the progressive gastritic lesion in p a Some investigators confirmed this view (*Meulengracht* 1939 *Cox* 1943) while others (*Magnus & Ungley* 1938) did not find evidence of inflammation but solely a severe atrophy

The introduction of the flexible gastric biopsy tube in 1949–50 (*Wood et al* 1949 *Tomenius* 1950) facilitated the study of the gastric mucosa and it was by this time recognized that the gastric lesion in p a was directly related to the pathogenesis of this disease (*Castle* 1929)

The results obtained by various workers using biopsy technique in the study of p a have not resolved the problem which histological picture is typical in this disease In 39 out of 44 patients *Wood* (1951) classified the lesion as gastric atrophy In a later study by his group the incidence of gastric atrophy was reported to range at 40 per cent (*Joske et al* 1955) while the remaining 60 per cent (of 100 patients) showed various degrees of atrophic gastritis In a series of 16 p a pa

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tients *Markson & Davidson* (1956) found gastric atrophy in 14 patients *but included in this group 4 patients with a slight cellular infiltration* Thus only 2 out of the 16 patients were classified as cases of atrophic gastritis Contrary to these findings *Williams et al* (1958) found chronic atrophic gastritis in 38 out of 44 patients but these authors remarked that the cellular infiltration was slight in 17 among the 38 patients and concluded that well marked infiltration with inflammatory cells occurred in at least half of the patients 37 among these 44 patients had intestinal *metaplasia and in 14 of the biopsies* parietal cells were present

Siurala et al (1960) found in 50 patients with p a that inflammatory infiltration was present in 38 intestinal metaplasia in 32 and pseudopyloric glands in 40 According to the latest biopsy studies of p a (*te Velde et al 1966 Wright et al 1966*) gastric atrophy is found to be present in approximately one half of the patient atrophic gastritis *being manifest in the other half*

Whether gastric atrophy or atrophy gastritis should be considered the typical lesion in p a has been an object of much discussion probably due to the differing definitions of these two conditions As remarked by Coghill Everyone working in this field has his own classification

We have analysed the data derived from histological studies on gastric biopsies from p a patients and will discuss in detail the criteria required for a classification of the pathological changes in an attempt to make these criteria more objective

MATERIAL AND METHODS

43 patients with p a in remission were studied The diagnosis had been established by *findings such as megaloblastic anemia* low serum vitamin B_{12} a pathologically low absorption of vitamin B_{12} correctable by oral hog intrinsic factor preparations and besides that the patients anaemia had responded favourably to treatment (usually by a vitamin B_{12} depot preparation) In most patients an augmented histamine test was performed as well which in all cases revealed achlorhydria The patients were 28 women and 15 men The mean age was [illegible] years (range 37–82) and the mean duration of the disease (time since the diagnosis was established) was 4 years (range 0–29)

Each patient had a single gastric biopsy taken from the corpus fundus part of the stomach The biopsy was performed using a Crosby capsule (*Crosby & Kugler* 19[illegible]7) which in all cases was sited under fluoroscopic control

All biopsy specimens were immediately fixed in Lillies AAF buffered neutral formol solution or formol-calcium and embedded in paraffin or gum arabic sucrose

The sections were stained with haematoxylin eosin PAS and by the method of *Mark s & Drysdale* (1947) with added *Alcian blue and studied in the light microsc* [illegible]

RESULTS

Pathological changes were found in all biopsies

The incidences of 13 different histological findings are summarized in Table 1 Five of these were taken as particularly important ones The thickness of the mucosa and the presence or absence of a) parietal cells b) intestinal metaplasia c) *pseudopyloric glands* d) inflamma

TABLE 1

Pathological Changes in Gastric Biopsies from 43 Patients with Pernicious Anaemia

Finding		n (%)	Combined
Mucosal thickness			
	below 500 μ	14(36%)	34 (87%)
	between 500μ and 800 μ	20(51%)	
	more than 800 μ	5(13%)	
	undeterminable	4	
Structure of fundic glands			
	partly preserved	16(37%)	
	not preserved	27(63%)	
Parietal cells			
	absent	30(70%)	
	present	13(30%)	
Chief cells			
	absent	37(86%)	
	present	6(14%)	
Intestinal metaplasia			
	absent	11(26%)	
	slight	14(32%)	32 (74%)
	dominating	18(42%)	
Pseudopyloric glands			
	absent	5(12%)	
	present	38(88%)	
Fibrosis and thickening of muscularis mucosa			
	absent	9(21%)	
	present	30(70%)	
	undeterminable	4(9%)	

Finding		n (%)	Combined
Inflammatory infiltration with lymphocytes and plasma cells			
inconspicious	absent	1(2%)	13(30%)
	slight	12(28%)	
conspicious	pronouced	26(60%)	30(70%)
	heavy	4(10%)	
Lymphocyte aggregations with or without germinal centres			
	absent	19(44%)	
	present	24(56%)	
Inflammatory infiltration predominantly in and around muscularis mucosa			
	absent	21(49%)	
	present	18(4[illegible])	
	undeterminable	4(9%)	
Russell bodies			
	absent	33(77%)	
	present	10(23%)	
Polymorphnuclears in lamina propria			
	absent	39(91%)	
	present	4(9%)	
Eosinophils in lamina propria			
	absent	39(91%)	
	present	4(9%)	

tory infiltration with lymphocytes and plasma cells It is noteworthy that only 36 per cent showed a mucosal thickness below 500μ that parietal cells were present in 30 per cent and that only one biopsy showed complete absence of inflammatory infiltration The percentages of intestinal metaplasia pseudopyloric glands and conspicious inflammatory infiltration were high (74 per cent 88 per cent and 70 per cent)

The combined incidences of the five most important histological findings (vide supra) are given in Table 2 Half of the 14 patients with a very thin mucosa had a conspicious inflammatory infiltration and this was found in 22 out of the 34 biopsies in which the mucosal thickness was seen to be reduced As expected absence of parietal cells was most frequent in the biopsies revealing a very thin mucosa

In order to seek out the most typical appearance of the gastric mucosa in p[illegible] it was calculated how often three of the following five findings concomitantly occurred in the biopsy a) pseudopyloric glands b) intestinal metaplasia c) conspicious inflammatory infiltration d) mucosal thickness below 800μ and e) abs[illegible] of parietal cells (Table

TABLE

Combined Incidence of 2 Important Histological Findings in

		Mucosal thickness				
		No	500 μ >	500 μ < 800 μ >	800 μ <	undeterminable
Mucosal thickness	Number	43	14	20	5	4
	< 500 μ	14				
	> 500 μ < 800 μ	20		-		-
	> 800 μ	5				
	underterminable	4				-
Parietal cells	absent	30	12	12	2	4
	present	13	2	8	3	0
Intestinal metaplasia	absent	11	3	3	3	2
	slight	14	5	8	1	0
	dominating	18	6	9	1	2
Pseudopyloric glands	absent	5	2	2	0	1
	present	38	12	18	5	3
Inflammatory infiltration with lymphocytes and plasmacells	absent	1	1	0	0	0
	slight	12	6	5	1	0
	pronounced	26	6	14	3	3
	heavy	4	1	1	1	1

3) The combined incidence of c + d + e and b + c + e are especially noteworthy

The mucosal thickness was below 800μ in 34 out of the 39 biopsies where this could be determined (Table 2) 24 of these lacked parietal cells in 11 of these 24 biopsies absence of or inconspicious inflammatory infiltration was noted and 6 of these showed furthermore intestinal metaplasia In Table 4 this sequence is shown for all of the 34 biopsies with a mucosal thickness below 800μ and also the sequences for the 2 sub groups of biopsies below 500μ and between 500 and 800μ

In Table 5 is shown the relationship between the incidence of the five important histological findings and the patients age at the beginning of the disease together with the duration of the latter Although no clear cut conclusions could be drawn from these figures it seems that a very thin gastric mucosa and inconspicious inflammatory infiltration was most frequently noted in patients in the group of elderly individuals

By way of conclusion it can be stated that the degree of histological abnormalities varied considerably Fig 1 and Fig 2 demonstrate the extremes observed in this series of gastric biopsies from 43 patients with p a

2

Gastric Biopsies of 43 Patients with Pernicious Anaemia

Parietal cells		Intestinal metaplasia			Pseudopyl glands		Inflammatory infiltration			
absent	present	absent	slight	dominating	absent	present	absent	slight	pronounced	heavy
30	13	11	14	18	5	38	1	12	26	4
8	3									
9	5									
13	5									
3	2	1	1	3						
27	11	10	13	15						
1	0	0	0	1	0	1				
11	1	4	3	5	1	11				
17	9	5	10	11	2	24				
1	3	2	1	1	2	2				

DISCUSSION

Badenoch & Richards (1955) reported that the degree of atrophy of the specific glandular tissue in gastric biopsies from their 15 p a patients was not greater in those in whom the condition had been present for a long time *Williams et al* (1958) confirmed this and found no relation ship between the mucosal appearance and the ages of the patients Our findings (Table 5) confirm these previous reports although changes seen to have a slight tendency to be more severe in the group of elderly patients

In the evaluation of gastric biopsies the term atrophy denotes A) a thinning of the gastric mucosa and B) a disappearance of the specific glandular tissue (i e parietal cells)

The term atrophy is included in the descriptive diagnoses atrophic gastritis and gastric atrophy where the former signifies an atro phic mucosa *with*, the latter *without* inflammatory infiltration

If only a single criterium is used (thinning of the gastric mucosa absence of parietal cells presence or absence of inflammatory infil tration) the incidence of gastric atrophy (Table 1) varies from 2 to 87 per cent and the incidence of atrophic gastritis varies from 10 to 98 per cent in p a patients

If the combination gastric mucosa below 800μ the total disappear

TABLE 3

Combined Incidence of 3 Important Histological Findings in Gastric Biopsies of 43 Patients with Pernicious Anaemia

a	Pseudopyloric glands present	88%	+	+	+			+	+	+		
b	Intestinal metaplasia present	74%	+			+	+	+	+		+	
c	Conspicious inflammatory infiltration present	70%			+		+	+		+	+	+
d	Gastric mucosa below 800 μ	87%	+	+	+	+	+					+
e	Parietal cells absent	70		+		+			+	+	+	+
	Incidence of combination		62%	56%	49%	49%	49%	47%	42%	37%	33%	33%

TABLE 4

39 Gastric Biopsies in Which Mucosal Thickness was Measurable

Mucosal thickness	below 500 μ	between 500 μ and 800 μ	below 800 μ	
Number	14 (36%)	20 (51%)	34 (87%)	biopsies
Among these parietal cells were absent in	↓ 12 (31%)	↓ 12 (31%)	↓ 24 (62%)	biopsies
Among these inconspicious inflammatory infiltration was found in	↓ 7(18%)	↓ 4 (10%)	↓ 11 (28%)	biopsies
In this last group intestinal metaplasia was found in	↓ 5 (13%)	↓ 1 (2%)	↓ 6 (15%)	biopsies

TABLE 5

Age of Patients and Duration of Pernicious Anaemia in Relation to Gastric Biopsies Findings

Duration of pernicious anaemia		Mucosal thickness				Parietal cells		Intestinal metaplasia			Pseudo pyloric glands		Inflammatory infiltration			
		< 500 μ	500 μ–800 μ	> 800 μ	undeterminable	absent	present	absent	slight	dominating	absent	present	absent	slight	pronounced	heavy
	No	14	20	5	4	30	13	11	14	18	5	38	1	12	26	4
0–3 year	20	9	6	3	2	15	5	4	6	10	3	17	0	7	10	3
4 year or more	23	5	14	2	2	15	8	7	8	8	2	21	1	5	16	1
Age at debut of symptoms																
30–59 year	17	4	8	3	2	10	7	6	4	7	2	15	1	2	11	1
60 year or more	26	10	12	2	2	20	6	5	10	11	3	23	0	10	15	1

ance of parietal cells and conspicious inflammatory infiltration is taken as necessary criteria for *very severe atrophic gastritis* then a presence of this condition was apparent in 33 per cent of the biopsies (Table 3)

If a mucosal thickness below 800μ absence of parietal cells and inconspicious inflammatory infiltrations taken together constitute the criteria for gastric atrophy this is found in 28 per cent while the incidence of this condition is reduced to 18 per cent if only biopsies with a thickness below 500μ are included (Table 4) Intestinal metaplasia is a frequent finding in p a (*Williams et al* 1958 *Siurala et al* 1960) (Table 1) If this phenomenon is included in the criteria of gastric



Fig 1

A representative part of the biopsy showing least atrophy A considerable number of parietal cells are preserved The patient had achlorhydria
Haematoxylin eosin × 100 (*Inset* × 400)

atrophy the incidence of this condition is reduced to 15 per cent and 13 per cent respectively (Table 4)

It should be noticed however that only one biopsy was completely without inflammatory infiltration so that the incidence of gastric atrophy would be much lower if slight inflammatory infiltration would preclude this diagnosis

Furthermore the relatively great number of biopsies showing only slight inflammatory infiltration demonstrates that the distinction between atrophic gastritis and gastric atrophy is not very marked as

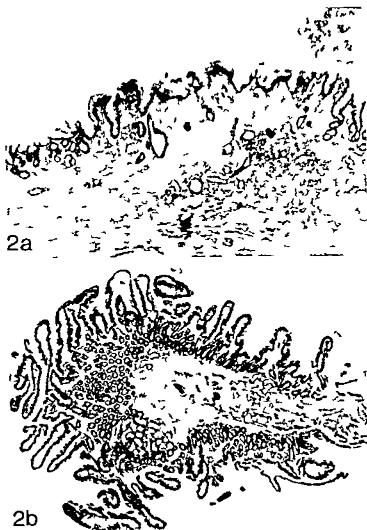


Fig 2

Complete atrophy of the specific glandular tissue A Representative part of biopsy without intestinal metaplasia Haematoxylin eosin × 40 B Total intestinalization with villous formation and pseudob unner glands (Another biopsy in the material had a similar appearance) Haematoxylin-eosin × 25

observed also in other series of gastric biopsies from non p a patients (*Shiner & Doniach* 1957 *Christiansen & Johansen* 1966)

It should be emphasized that no specific changes were disclosed in these biopsies The appearance of the mucosa was very close to that found in biopsies taken from patients without p a and showing severe grades of atrophic gastritis (*Christiansen & Johansen* 1966) These findings confirm the opinion held by *Mac Donald & Rubin* (1967) that gastric biopsy is without diagnostic value in pernicious anaemia

Applying the above mentioned criteria very severe atrophic gastritis and gastric atrophy, a considerable number of the biopsies were left in which various degrees of atrophic gastritis were found. This underline the remarkable variability of the pathological changes affecting the gastric mucosa in this condition in which depression of the gastric function is most severe.

SUMMARY

Data derived from histological studies of gastric biopsies from 43 patients with pernicious anaemia were analysed with respect to the typical histological appearance in this condition. The criteria for a classification of the biopsies are discussed since a great variation in the degree of pathological changes was observed. A very severe atrophic gastritis was found in 33 per cent and according to the criteria used gastric atrophy was found in 28 per cent to 2 per cent. The remaining biopsies showed varying degrees of atrophic gastritis. Compared with the histological changes in biopsies from non p a patients with gastritis no specific changes were found.

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CELL POPULATION KINETICS OF THE MOUSE EAR EPIDERMIS FOLLOWING APPLICATION OF 3 METHYLCHOLANTHRENE

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In a previous study the cell population kinetics in untreated hairless mouse epidermis and in epidermis treated with one single application of some chemical agents were investigated by studying the disappearance of H^3-DNA from the skin (*Skjæggestad* 1964) The back skin of hr/hr mice was used Cell nuclei in DNA synthesizing phase were labelled in vivo with radioactive thymidine (H^3T) by means of an intraperitoneal injection In this way some cell nuclei contain a certain amount of radioactive material (H^3 DNA) This radioactivity assuming it is stable remains in the epidermis until the labelled cells or their descendants are lost from the surface If no new radioactivity enters the population during the experimental period the amount of label found in the epidermis at different times after the H^3T injection is thus an index of the rate of cell loss Pieces of epidermis were separated from the skin by means of a special keratotome weighed and dissolved in Hyamine The H^3 activity was measured by means of the liquid scintillation technique The area of the separated epidermis was very difficult to measure exactly and the activity therefore had to be related to the weight of the specimen The volatile activity due to H^3OH in the wet specimen was subtracted according to an estimated H^3OH half life curve During the cutting procedure varying amounts of corium adhered to the specimen The activity observed thus originated both from the epidermis and the corium The activity in the corium however was found to be comparatively small and the method was clearly valid for measuring the kinetics of the normal epidermis When the epidermis was injured by chemical agents however the validity of the method was burdened with more uncertainties and in some of the experiments *an increase in the total radioactivity the first days after the labelling* was seen The cause of this increase was assumed to be an invasion of radioactive cells into the corium where an inflammatory reaction is always seen following application of methylcholanthrene (MCA) to the epidermis By this method alone however it was difficult

to demonstrate that the increase of activity originated from this invasion of cells especially because other uncertainties concerning the method also existed The aim of the present work therefore has been to repeat some of the experiments by another method where some of the earlier uncertainties have been eliminated In addition the autoradiographic technique has been used

MATERIALS AND METHODS

Ear epidermis of the hr/hr strain of mice has been used The animals were 10–16 weeks old and the weights ranged from 23 to 32 grams The cell nuclei were labelled through intraperitoneal injection of 50 μC H^3 thymidine (H^3T) between 8 00 a m and 9 00 a m Two drops of a carcinogenic agent 1 per cent MCA in benzene solution were applied to both sides of the ears by a Pasteur pipette either 3 days before or 4 days after the H^3T injection At different time intervals following the injection the ears were removed and immediately anchored on a cork block. By use of a cylinder shaped metal tube with a sharp edge a circular part of the ear was punched out In this way ear specimens with constant diameter (0 9 cm) and a total epidermal area of about 1 25 sq cm were obtained

For measurement of the non volatile H^3 activity the specimens were dried at 70 C for 20 hours and then dissolved in 1 5 ml Hyamine 10 X at 55 C for 36 hours A liquid scintillator was later added to the tissue solution and the total activity counted in a Tri Carb Liquid Scintillation Spectrometer The background activity was subtracted and correction for quenching by use of the internal standard technique was performed The results were expressed as total activity per ear specimen

For the autoradiographic investigation about 5 μ thick histological sections were made of paraffin embedded ears and the stripping film technique was used. In each slide 20 vision fields (× 800) from each cell population (epidermis and corium) were investigated. The results were expressed as number of labelled cells per vision field According to the grain counts the labelled cells were designated as markedly (≥ 20 grains) moderately (11–19 grains) and slightly (6–10 grains) labelled Cells were not considered as labelled if they contained less than 6 grains over each cell nucleus

RESULTS AND COMMENTS

Problems Concerning the Method

The size of the ear specimens was tested by weighing them on a Mettler Analytical Balance The results are seen in Table 1 the mean weight of the specimen is 8 2 mg The variations are moderate (SD = 0 8 mg) and when specimens taken from the same mouse are compared as in the present study SD is only 0 45 mg With the method used it is improbable that the variations of the areas of the specimen are greater than the variations of the weights In the expression of the results as activity per ear specimen the possible errors due to differences in the size of the specimen are therefore satisfactorily small

The ear epidermis consists of one to three layers of epithelial cells the outside a little thicker than the inside Abortive hair follicles are seen at irregular intervals especially on the outside At the basis of the ear this difference between the outside and the inside is greater but with the present method where the most peripheral part of the ear is punched out this basic part is not involved The average number of epithelial cells per microscopic vision field 800) of interfollicular

MCA painted than in the normal specimen but both values are constant for at least 48 hours

Labelled cells have been counted both in the normal and in the painted ear specimen by means of the autoradiographic technique When Fig 3 A and B are compared it is also seen that the number of labelled cells both in the epidermis and in the corium is much higher in the MCA painted than in the normal ears

The constant value reached one hour after the H^3T injection represents the socalled incorporation value and *remains constant for* at least 2 days The high non volatile activity found 5 to 30 minutes after the H^3T injection is probably due to H^3T and H^3T phosphates not bound to DNA in the specimen during the incorporation To prevent erroneous incorporation values investigations of this type must therefore not start earlier than one hour after the H^3T injection The incorporation value is higher in the painted than in the normal ear specimen Judged from the autoradiographic investigation this is probably due to an increased activity in both the corium and the epidermis

The Normal H^3 DNA Disappearance Curve

In the epidermis it is necessary to consider both the dividing process going on among the basal cells and the maturing process going on among the differentiating cells The latter are gradually transformed to keratin and shed from the surface The average time period elapsing between two subsequent mitoses in the same basal cell is called the mean generation time The term turnover time indicates the average *time period elapsing from the formation of a differentiating cell* until it loses its nucleus and is transformed to keratin (*Iversen & Evensen* 1962) In which way the differentiating cells are formed has been much discussed (*Iversen & Bjerkness* 1963 *Leblond et al* 1964 *Oehlert* 1966 *Iversen & Bjerknes* 1968) On the average however half of the newly formed cells move outward to keratinize while half remain in the basal layer The different time parameters in mouse epidermis have such values that after a single injection of H^3T when a certain amount of basal cells are labelled and some time after these cells have passed mitoses about half of the activity will remain in the basal cells while the other part is in the maturing differentiating cells The total activity *measured in he epidermal population will remain constant until la*belled differentiating cells have lost their nuclei and the activity thus released (*i e* reabsorbed or shed with the horny layer) is lost The period of constant activity thus depends mainly upon the turnover time of the differentiating cells and the decrease of activity depends mainly upon the speed of cell division in the basal layer *i e* the mean generation time In the autoradiographic investigation the number of labelled cells will be doubled following cell division while the grain count as an expression of the activity per cell nuclei will be halved When the

grain count becomes below 6 grains per cell nucleus these cells will not be counted as labelled cells because they cannot be distinguished from accidental background activity Some earlier moderately and all slightly labelled cells will therefore no longer be recognized as labelled cells after mitosis

TABLE 2

The Non Volatile H^3 activity in the Ear Specimen at Different Time Intervals after H^3T Injection and MCA Application The two Ears of the same Mouse have been Compared by Removing them at Different Times after the H^3T Injection

Days after injection of H^3T	No of mice (pair of ears)	Arith metic mean	$2\,SD_{(\bar{X})_n}$	Per cent of initial value
The untreated ear specimen				
Ratio H^3 activity 4th day/1st day	9	0 99	0 07	99
Ratio H^3 activity 6th day/4th day	9	0 85	0 03	84
Ratio H^3 activity 9th day/6th day	9	0 95	0 08	80
Ratio H^3 activity 15th day/9th day	9	0 82	0 0	65
Application of MCA 4th day after H^3T injection				
Ratio H^3 activity 6th day/4th day	5	0 82	0 06	81
Ratio H^3 activity 9th day/6th day	16	0 87	0 04	70
Application of MCA 3 days before H^3T injection				
Ratio H^3 activity 4th day/1st day	12	1 14	0 07	114
Ratio H^3 activity 6th day/4th day	9	0 80	0 06	91
Ratio H^3 activity 12th day/6th da	9	0 69	0 04	65

The disappearance of non volatile H^3 activity from unpainted ear specimens following injection of H^3T has been investigated Each mouse has been used as its own control *i e* the activity in the one ear has been compared with that in the other ear 4 days after the injection by removing one ear on the 1st day and the other ear on the 4th day In the same manner the activity 4 and 6 days 6 and 9 days and 9 and 15 days after the injection has been compared The results are seen in Table 2 and Fig 2 A The activity is constant the first 4 days followed by a gradual decrease in activity reaching 65 per cent of the initial value at the 15 day

The same experiment has been performed with regard to the 4th and the 15th days by means of the autoradiographic technique The results are seen in Fig 3 A It is seen that the number of labelled cells in epidermis decreases considerably at the same time as markedly and moderately labelled cells have nearly disappeared from the population In the corium on the contrary no decrease in the total number of labelled cells is seen and the distribution of the grain counts is about the same

The disappearance curve from the ear specimen may depend upon loss of activity from the corium as well as from the epidermis As men

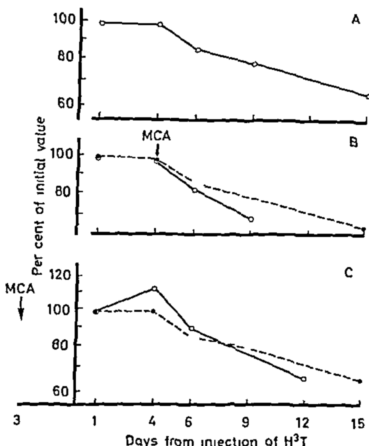
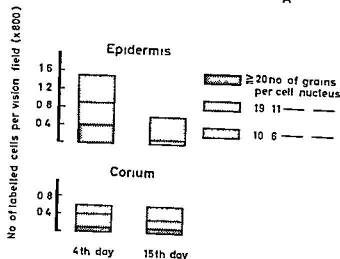


Fig 9

A The non volatile H³ disappearance curve in normal ear specimen B The non volatile H³ disappearance curve in ear specimen painted with MCA 4 days after the H³T injection (———— the normal curve) C The non volatile H³ disappearance curve in ear specimen painted with MCA 3 days before the H³T injection (——— the normal curve)

tioned above and confirmed from the autoradiography cell loss is fairly more rapid in the epidermis than in the corium The decrease in activity is therefore mostly due to loss from the epidermis alone The activity in the corium represents a nearly constant but unknown magnitude of activity The time period elapsing between labelling and mitosis is comparatively short and will interfere little with the results The period of constant activity therefore represents the time elapsing from the formation of differentiating cells until the activity originating from these is lost from the population i e the minimum turnover time of the differentiating cells in ear epidermis is at least 4 days A study of the ear epidermis by the Colcemid method has shown (*Iversen* 1967) that there is on the average 70 per cent basal cells and 30 per cent differentiating cells in this tissue The mean generation time of the



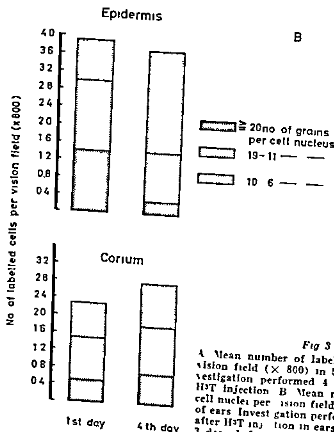


Fig 3

A Mean number of labelled cell nuclei per vision field (× 800) in 5 pairs of ears Investigation performed 4 and 15 days after H³T injection B Mean number of labelled cell nuclei per vision field (× 800) in 6 pairs of ears Investigation performed 1 and 4 days after H³T injection in ears painted with MCA 3 days before the injection

basal cells is 16 days The mean turnover time of the differentiating cells is thus $(16 \times 30)/70 \approx 7$ days

The further course of the disappearance curve as mentioned above depends upon the mean generation time of the basal cells This value could be calculated from the steepness of the first falling part of the line if the magnitude of activity in the corium was known On the other hand the activity in the corium can be determined when the mean generation time of the basal cells is known In 1967 Fagering found as mentioned above that this mean generation time is 16 days Using this value the steepness of the line will be correct if an amount of activity corresponding to 20 per cent of the initial one is subtracted from all figures *i e* the first 4 days after H^3T injection 80 per cent of the total non volatile H^3 activity in the ear specimen originates from epidermis while the remaining 20 per cent rests in corium

The Effect of Methylcholanthrene (MCA)

The H^3 DNA disappearance curve following application of MCA has been investigated In one experiment the application of MCA was given 4 days after the H^3T injection *i e* at the moment the activity starts to decrease The results are seen in Table 2 and Fig 2 B The MCA application has provoked a faster decrease in activity *i e* an initial increased cell loss From the steepness of the line the rate of cell loss can be calculated to at least twice the normal during the experimental period *i e* the first 5 days after the MCA application

In another experiment the application of MCA was given 3 days before the H^3T injection *i e* the disappearance curve was followed from the moment when the ear had been under the influence of a single MCA application for 3 days The results are seen in Table 2 and Fig 2 C In contrast to the normal disappearance curve *the activity increases from the 1st to the 4th day after the H^3T injection* Later the decrease in activity is faster than normal It has earlier been shown (Fig 1) that the incorporation value is higher under these experimental conditions The proportion between the magnitude of activity in the corium and the epidermis may be another than in the untreated ear specimen and therefore unknown At no events however the corium activity can be more than 50 per cent

The same experiments have been performed by means of the autoradiographic technique The results are seen in Fig 3 B Labelled cells are found both in the epidermis and in the corium In the epidermis a pronounced decrease in number of markedly labelled cells is seen as well as a decrease also in moderately labelled cells The slightly labelled cells have increased in number during the same period In the corium a slight increase in markedly moderately as well as slightly labelled cells is seen

From the last experiment it is seen that the total non volatile activity

has increased *i e* new activity has invaded the ear specimen between 1 and 4 days after the H^3T injection It has already been shown that the activity was constant from the 1st to the 2nd day indicating that new activity comes into the skin from the 2nd to the 4th day In the autoradiographic part of the experiment an increase in the number of labelled cells has only been found in the corium The pronounced decrease in markedly labelled cells in the epidermis during a period of constant hyperplasia (Table 1 5–9 days after MCA) indicates a high rate of cell divisions in the epidermis (*Figjo* 1966) The unchanged total number of labelled cells in spite of this also indicates a heavy cell loss In the corium however there is no reduction in the grain counts suggesting no or very few cell divisions The increase in the total number of labelled cell nuclei must therefore be caused by an invasion of labelled cells probably leucocytes and histiocytes From the autoradiographic specimen with film emulsion covering the sections it is well nigh impossible to judge from the morphology of the cells whether they are lymphocytes or histiocytes but most of them look like rather large histiocytes or fibroblasts

GENERAL DISCUSSION

One of the methods used in the present study rests upon measurement of non volatile H^3 activity in a constant area of ear specimen The disadvantage is that activity from the corium interferes with the results In an earlier study (*Skjæggestad* 1964) the same parameters have been investigated on back skin by a method where the corium constituted a smaller part The normal disappearance curve was therefore easier to interpret by this method and the application of MCA gave more distinct results This earlier method was however burdened with other methodological uncertainties and the increase in activity found in some experiments was more difficult to interpret Comparing the results found by the two methods a very good correlation is seen This gives strong support to the statement that the following conclusions are valid

i) *Application of MCA causes an initial increased loss of cells from epidermis*
ii) *Three days after application of MCA new endogenous H^3 activity enters into the skin*

Two main factors can be discussed in connection with the latter

A Local re utilization of H^3DNA and its breakdown products B A flow of labelled migratory cells into the skin

A Re utilization can theoretically be local *i e* from the dead cells in the same population or general The former is of special interest in the epidermis since the fate of the DNA and its breakdown products during keratinization is uncertain (*Pelc* 1959 *Mercer* 1961) In an earlier

study the H^3 activity passing through the horny layer was found to be equivalent to the H^3 activity which had passed through the epidermis (*Skjæggestad* 1964). Re-utilization of significant extent in the epidermis of our hairless mice is therefore improbable.

A general re-utilization especially from the gastro-intestinal and the erythroid cells has been discussed (*Steel & Lamerton* 1965). *Steel* (1966) found a systemic re-utilization in tumours with a very high growth rate (initial volume doubling time of about 30 hours). In tumours with doubling time of 7–8 days and 14 days however no detectable re-utilization was found. From these investigations he claims that a tumour must have a reasonable high growth rate during the period of high availability of H^3-labelled breakdown products if re-utilization shall take place. In the epidermis of hairless mice an increase in the growth rate is observed 5–7 days after application of MCA (*Elgjo* 1966), i.e. in the same period as the increase in activity was found in this study. At this time H^3 labelled breakdown products may also be available. According to *Steel* (1966) the re-utilization should be highest where the growth rate is highest. From the autoradiographic investigation however, the increase in activity is located only to the corium indicating that if there is any re-utilization it is found in the corium where the growth rate is very slow.

B. The second factor which might contribute to the observed increase of label is a flow of labelled cells into the specimen during the experimental period. Circulating leucocytes are most likely to cause a significant transfer of H^3 label. In a normal ear specimen where the condition is that of an even distribution of leucocytes throughout the animal this effect is probably small and unimportant. Following application of MCA a migration of labelled cells due to the inflammatory reaction may appear as a wave of activity entering into the population. *Montgomery et al.* (1965) found 3 and 4 times as many labelled round cells in healing wounds as in the control biopsies. They also found increase in the number of labelled fibroblasts in accordance with *Lichtelin & Linna* (1967) who also have shown that thymus lymphocytes DNA may be re-utilized for cell proliferation in healing wounds. Cells previously labelled and later entering into areas of inflammation have also been found by other authors (*Gillman & Wright* 1966).

Application of MCA provokes an inflammatory reaction especially in the corium. *Montgomery et al.* (1965) observed that the number of labelled round cells progressively increased in a wound section from the 1st to the 8th day of healing. During the period in which an increase in activity in my experiments is measured, i.e. 5–7 days after application of MCA, new inflammatory cells most probably enter the specimen. Since H^3T has been injected 2 days before this period many of these leucocytes must be labelled. The results from the autoradiographic investigation give a strong support to this assumption because an increase in number of labelled cells is found in the corium during

this period It is however difficult to judge how far this is responsible for the whole increase in activity It may be too small to explain the total increase in activity or more probably it may be so pronounced that a small decrease in epidermal activity is camouflaged In an earlier investigation (*Skjæggestad* 1964) a *decrease* in activity was found during the same period when MCA was applied 1 and 2 days before the H^3T injection But as in the present study an *increase* in activity was found when the MCA was applied 3 and 4 days before the injection The degree of invasion of labelled cells into the corium or the extent of possible re utilization during the experimental period is uncertain but probably equal in specimens labelled 1–2 and 3–4 days after the MCA application A shift in the population kinetics thus must have taken place Cells labelled 1 and 2 days after the application of MCA are lost at rate that is fairly more rapid than that at which cells labelled 3 and 4 days after the application are lost i e the initial increased cell loss has ceased This change in population kinetics is seen at about the same time as the hyperplasia begins (*Iversen & Evensen* 1962) and thus even supports the theory that the hyperplasia is due to this population shift with an epidermis consisting of newly formed cells with a long life expectancy

SUMMARY AND CONCLUSIONS

In an earlier study the cell population kinetics of the normal hairless mouse epidermis and of epidermis influenced by one single application of different chemical agents was investigated by studying the disappearance of H^3 DNA from the population This method is burdened with some uncertainties and the interpretation of some of the results was therefore difficult In the present study some of the earlier results have been re examined by a new and simpler method The non volatile H^3 activity has been measured in a constant area of ear skin About 80 per cent of this activity originates from the epidermal population while the remaining 20 per cent rests in corium In addition the autoradiographic technique has been used The results found by this new methods were in agreement with the earlier results and support the conclusions as follows

A single application of 1 per cent methylcholanthrene in benzene solution (MCA) to the epidermis causes an initial increased cell loss which at least is twice the normal during the first 5 days When the H^3T injection is given 3 days after the MCA has been applied to the epidermis an invasion of labelled cells into the corium layer is seen The degree of this invasion which may interfere with the results is difficult to estimate

There are good indications that the initial period of increased cell loss after a single application of MCA is followed by a secondary period of decreased cell loss leading to a transient hyperplasia

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THREE CASES OF POSTLACTATIONAL BREAST TUMOUR OF A PECULIAR TYPE

By

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Benign unilateral enlargement of the female breast is not common in the Swedish population *Strombeck* (1964) reported unilateral hyperplasia in six of 1042 women operated upon for macromastia Various causes of macromastia have been reported cystosarcoma phyllodes (*Treves & Sunderland* 1951) macromastia during pregnancy also called gigantomastia (*Lewison et al* 1960) giant fibro adenoma (*Jordal & Sorensen* 1961) and puberty or virginal hypertrophy (the most common diagnosis in *Strombeck s* material) All these types except fibroadenoma and cystosarcoma phyllodes are usually bilateral This paper reports three cases of a tumourlike and extensive growth in one breast after lactation The macro and microscopical picture was of a peculiar type and as far as we have found not hitherto described

CASE REPORTS

Case 1

B J A 40 year old woman Normal deliveries in 1945 and 1957 The left breast had since puberty been somewhat larger than the right After the last lactation period in 1957 the left breast started to increase substantially in size and continued to do so for a couple of years but then persisted unchanged until 196[illegible] when the patient sought medical advice and was referred to our service of plastic surgery

On admission the left breast was found to be four times the size of the right one which was normal in size and shape (Figs 1 a) The enlarged left breast was firm and the skin was bluish red with distended superficial vessels No tumour could be palpated with certainty and no discharge could be expressed Regional lymph nodes were barely palpable

At operation an incision was started in the submammary fold and the glandular tissue was exposed This tissue was incised and an enormous well-defined tumour was easily enucleated from the surrounding compressed breast tissue The remnants of the gland were sewn together with plastic reduction of the skin The post operative course was uneventful and the primary cosmetic result was acceptable At review four years later there were no signs of a recurrence The patient was content with the result of the operation and did not wish any further plastic correction (Fig 1 b)

The specimen (Fig 2) weighed 1400 g and had a diameter of 17 cm and thickness of 9 cm It was strikingly firm and well [illegible] with [illegible] thin fibrous



Figs 1 a and 1 b
Case 1 Before and after operation

capsule The cut surface was yellowish white with multiple cysts up to one centimeter in diameter and containing clear fluid

Histologic findings (Figs 3-5) There was an abundant dense fibrous stroma with collagen bundles and very few nuclei and unevenly distributed thin vessels with low endothelium The ducts were more or less widened with rather pr mi



Fig 2
Case 1 Cross specimen The cut surface is even pale with smooth walled cysts The margin is distinct

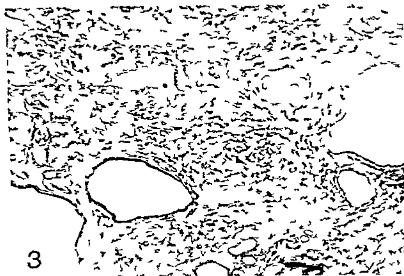


Fig 3

Case 1 Fibrous stroma with cysts atrophic acini and a few ducts van Gieson × 16

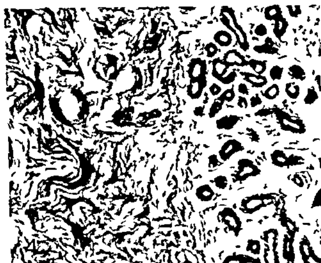


Fig 4

Case 1 To the left fib us stroma with collagen us bundles and to the right a group of rather normal acini with loose stroma van Gies n × 1?0

nent high cylindrical epithelium and a distinct myo epithelial layer Many cysts of varying diameter were seen some with high cylindrical epithelium others with a low single cell layer Some cysts had an epithelium with rich acidophil cytoplasm of apocrine type Scattered gland lobules were of ordinary appearance ith thin acini and a loose intralobular fibrillar strom M st of the lobules w e split up b thick collagenous bundles and showed part atrophied, partly cyst c glands There were no inflammatory cells In the perip as a small am nt of fat and



Fig 5

Case 1 Cysts with higher cylindrical epithelium of apocrine type Htx eosin × 120

a thin capsule like layer of fibrous tissue Some loose pieces of tissue received together with the large specimen consisted of normal skin and normal mammary tissue with lobules and strands of fatty tissue

Case 2

G H A 35 year old healthy woman After the second delivery and seven months lactation the left breast started gradually to increase in size and continued to do so for seven years before admission

On admission the left breast was about three times as large as the normal right one A tumour like firm lump twice the size of a fist was palpated It was not adherent to the skin Distended superficial vessels were seen The nipple was nor

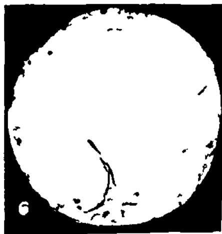


Fig 6

Ca e 2 Cr ss specimen Den cut u f with group s f small cy t



Figs 7–8

Fig 7 Case 2 Up to the left the well defined margin In the dense fibrous stroma lobules with thin normal acini and to the left a couple of cysts van Gieson × 16

Fig 8 Case 2 To the right fibrous stroma and to the left a lobule with dilated acini and dense stroma (cf Fig 4)

mal no discharge could be expressed and no regional lymph nodes could be palpated

At operation an incision was started in the submammary fold and the glandular tissue was exposed and a well defined tumour like mass was enucleated A regular mammaplasty according to Strombeck was performed The postoperative course was uneventful and the cosmetic result was acceptable at follow up

The specimen (Fig 6) weighed 840 g was 15 cm in diameter and 5 cm thick It was rather firm The cut surface was yellowish white with scattered small cysts containing clear fluid

Histologic findings (Figs 7–8) The picture was dominated by the dense fibrous stroma woth collagenous bundles with very few nuclei There were rather many thin vessels often with prominent endothelium Many gland lobules were of normal appearance with acini and loose intralobular stroma other lobules had atrophic ducts and acini and coarse intraacinar collagenous strands The larger ducts were not prominent Cysts were seen of varying diameter with mostly atrophic low epithelium The fibrous stroma contained some small strands of fatty tissue A thin peripheral layer of fibrous tissue covered the smooth surface

Case 3

(From another surgical service) A 20 year old healthy woman noticed an increasing swelling of the right breast during the last three months of her first gestation At the time of delivery the breast was markedly swollen tender and bluish It could not be suckled because of tenderness and had to be evacuated by pumping which was very painful The tumourlike swelling increased in size during the following eighteen months particularly during the last few months before surgical intervention

At operation a tumour of the breast could easily be enucleated It was round and well circumscribed The postoperative course was uneventful and the cosmetic result was good

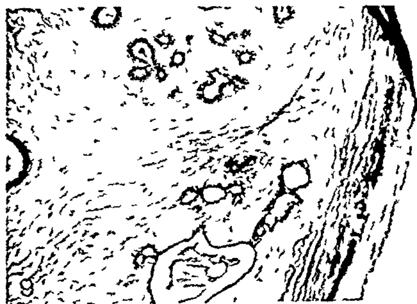


Fig 9

Case 3 The margin of the enucleated piece to the right It has a rather dense fibrous "capsule" Part of lobule with normal acini at top slightly dilated duct below Loose intra lobular stroma with stellate nuclei thick collagenous bundles between lobules. Htx eosin × 45

The specimen measured 7 × 7 × 5 cm its weight is not recorded The surface was smooth and the cut surface yellow white with small cysts Unlike what is seen in fibroadenoma it was dense and not soft and bulging

Histologic findings (Fig 9) There was a rich fibrous stroma with collagenous bundles and fairly numerous small and spindle shaped nuclei The lobules were all split up by strands of fibrous tissue and showed general dilatation of ducts and acini The epithelium was well preserved double layered and sometimes slightly folded The stroma around cysts was oedematous with thin fibres and more or less stellate nuclei and contained scattered lymphocytes

DISCUSSION

Macromastia has been described in association with clearly defined benign tumours or with diffuse hyperplasia of the glandular tissue Of the latter form two types are distinguished namely puberal or virginal hypertrophy and gestational hypertrophy also called gigantomastia Both types are usually bilateral but occasionally unilateral Histologically the hyperplasia is said to include the whole mammary tissue Gigantomastia usually disappears completely after the end of gestation There is no clinical or histological similarity between these types of macromastia and our cases

In some respects the microscopic picture in our cases resembled that of cystic disease (mastopathia cystica) But this diagnosis is vague and includes several types of changes In our cases the hyperplasia of the connective tissue without inflammation and the formation of cysts of varying size were the main features There was not much epithelial proliferation In contrast to findings in our cases cystic disease or mastopathia cystica is usually more diffuse and involves parts of the breasts without encapsulation

A rather well defined lesion of the breast is cystosarcoma phyllodes It is often unilateral and may affect the entire breast or only part of it The distinct and often highly cellular and florid histological picture of cystosarcoma phyllodes however rules out this diagnosis in our cases

Fibro adenomas of the breast are common and benign tumours They are said to be rarely more than 5 cm in diameter (*Geschickter* 1945) and are usually fairly well circumscribed The main elements of fibro adenomas are glandular and fibrous tissue in varying proportions with dissolution of the normal acinar and lobular structure In our cases the picture was dominated by the dense stroma with rather atrophic epithelium in normally organized acini and unevenly distributed cysts We also found some fat in the stroma which is not seen ordinarily in fibro adenomas The fibrosis resembled features described as involutionary alterations in the mammary parenchyma (*Bonser et al* 1961) but it was confined to the enucleated tumours in contrast to the ordinary surrounding breast tissue It was not the fibrosis with often compressed ductules which is seen in old fibro adenomas

Jordal & Sorensen (1961) describe their cases of unilateral macromastias as giant fibroadenomas The clinical history of their cases

closely resembled ours We have had the opportunity to study their microscopical slides and the structure was typical of fibro adenomas and did not resemble ours

We find that our three cases do not wholly agree with any of the described types of unilateral macromastia Their proper nature cannot be elucidated Perhaps the development after lactation may indicate an abnormal hormonal response

SUMMARY

Three cases of unilateral mammary enlargement starting in connection with pregnancy and lactation and operated upon with enucleation of large circumscribed tumours and with mammary plasty of remaining breast tissue are presented The two largest specimens weighed 1400 and 840 g respectively were well encapsulated and firm After enucleation a reduced mass of breast tissue and superfluous skin was surgically corrected in cases 1 and 2 The clinical course was uneventful The histologic picture was dominated by abundant fibrous stroma with slight cystic dilatation of ducts and acini which were otherwise normal

The differential diagnosis is discussed and the cases seem to represent a peculiar hitherto undescribed type of mammary tumour

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EFFECT OF ACTINOMYCIN D ON EPIPHYSEAL PLATE OF MICE

A Histological and ^{35}*S Autoradiographic Study*

By

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A variety of experimental methods are known by which the endochon dral growth may be disturbed in laboratory animals *e g* by hypo and hypervitaminosis by various kinds of hormonal disturbances by X ray irradiation or other ionizing irradiation as well as by plant enzymes or surgical intervention

The effect of actinomycin D on the endochondral growth of bone has however not been investigated Actinomycin D has a distinct molecular biological effect It selectively inhibits the DNA dependent synthesis of RNA and this effect of actinomycin D has been extensively used in ex perimental biological research (*Samuels* 1964) It was therefore con sidered of interest to find out to what extent actinomycin D produces morphological changes in the growth apparatus of the skeleton of ex perimental animals and what influence it has on the synthesis of chon droitin sulphuric acid in the epiphyseal growth zone

MATERIAL AND METHODS

Actinomycin D from MSD[1] was dissolved in physiological saline (1 mg/15 ml) $Na_2{}^{35}SO_4$ from Amersham was given in a solution containing 750 μCi/ml carrier free ^{35}S to a dose of 5 μCi/g bodyweight

8 white mice weighing about 15 g were used The animals were divided into three groups Groups I and II which consisted of each 10 animals were given actinomycin intraperitoneally Group I received actinom cin D in two doses of 1 μg/g body eight at the start and after 24 hours and was sacrificed 8 hours after the last dose Group II received a single dose actinomycin of 0 75 μg/g b dyweight and was sacrificed after 27 hours Group III consisted of 8 animals and served as control All three groups were given ^{35}S two hours befor death

The hind legs—from the middle of the femur to th middle of the tibia—were removed fixed for two days in 5 per cent formalin containing 0 5 per cent cetyl pyridine chloride (to prevent dissolution of the mucopol saccharides) (*Engfeldt & Hjertquist* to be publ) decalcified in a mixture f equal parts of 40 per cent

This investigation was made possible by research grants from the *Swed sh Medi al Research Council* (Project No K67–17X–??38–01)

[1] Courteously supplied by Merck Sharp & Dohme

formic acid and 20 per cent sodium citrate in distilled water dehydrated in an alcohol series and embedded in paraffin Some histological sections (5 6 μ thick) were stained with haematoxylin eosin and some according to van Gieson Unstained sections were used for autoradiography with stripping film Kodak AR10 The autoradiographs were exposed for 25 days after which they were developed for 5 minutes in Gevaert X ray developer G 230 rinsed in water a few seconds fixed for 5 minutes in Gevaert X ray fixer G 303 rinsed for 15 minutes in distilled water stained for 30 minutes with Mayer s haematoxylin only and then rinsed in water for 10 minutes The stained autoradiographs were dried in the air overnight dipped a few seconds in absolute alcohol and then mounted under a cover glass with Euparal This procedure proved to give the technically best autoradiograms

Histological sections treated in the same way but without radioisotope did not exhibit any darkening of the film emulsion

RESULTS

Preliminary studies had shown that in the large dose used in group I actinomycin will kill most of the animals within a few days In the present investigation the animals were therefore sacrificed 32 hours after the first injection The small dose in group II was not lethal

Histological Changes in the Epiphyseal Plate

The zones of the germinative and proliferative cells in groups I and II showed no change in arrangement of the cells compared with the control group The structure of the nuclei and cytoplasm also was normal The proliferation of the cartilage cells thus appeared to proceed undisturbed

The zone of hypertrophic cells in group I was however clearly thicker than normal (Figs 1a and 1b) The height of this layer was on the average 190 μ compared with about 90 μ in the control animals The hypertrophic cells in the entire layer were very uniform and did not show the gradual maturing or signs of degeneration seen in the lower most row of cells in the controls The nuclei were smaller and stained more intensely through the entire layer (Figs 1a and 1b) No pathological changes were observed in the hypertrophic layer in group II

Histological Changes in the Metaphysis

The cells between the primary trabeculae were sparser in group I than normally (Figs 2a and 2b) Between the trabeculae wide capillaries extended up to the growth cartilage and showed endothelial cells

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Figs 1–2

Fig 1a and b Microphotographs of epiphyseal plates of normal mouse (1a) and mouse treated with actinomycin as in group I (1b) In the latter long rows of hypertrophic cartilage cells with small dark nuclei Magnificati n × f3 Htx eosin

Fig 2a and b Microphotographs of the metaphyseal regions of normal mouse (2a) and mouse treated with actinomycin as in group I (2b) In Fig 2b only a small number of osteoblasts with flat nuclei Very few haematopoietic cells as compared with the control animal Magnification × 160 Htx-eosin


1A
1B
2A
2B

of normal appearance. Only a few fibroblasts were seen. Adjacent to the trabeculae was a relatively small number of osteoblasts whose nuclei were flattened compared with the cuboidal or rounded nuclei in the control animals (Figs 2a and 2b). The cytoplasm of the sparse osteoblasts was less basophilic than normally but had still the typical juxtanuclear vacuole (*Young* 1962). The trabeculae showed no signs of bone formation. Examination under higher magnification also showed several nuclei among the different cells undergoing karyorrhexis with intensely stained fragments of chromatin.

In the animals in group II the changes consisted only of a dilatation and congestion of the capillaries in the metaphysis. The bone forming cells had a normal appearance.

Further down towards the diaphysis the haematopoetic cells in the bone marrow in the animals in group I were sparser than normal. The animals in group II did not differ from the controls in this respect.

Autoradiographic Changes

The uptake of ^{35}S in the epiphyseal plate was lower in the animals treated with actinomycin (Figs 3 and 4). This was seen both in group I (two large doses of actinomycin) and in group II (a single smaller dose of actinomycin). As in the controls the activity was confined mostly to the cells in the germinative and proliferative layers of the epiphyseal plate and was seen mainly intracellularly.

DISCUSSION

The most striking change in the mice treated with a lethal dose of actinomycin was the decrease in the number of haematopoetic cells, osteoblasts and osteoclasts. The osteocytes already enclosed in intercellular bone substance and synthesizing protein at probably a low rate did not change morphologically. The reduction in the number of osteoblasts and osteoclasts may be due to the effect of actinomycin being so strong as to destroy those cells having a high rate of production of protein.

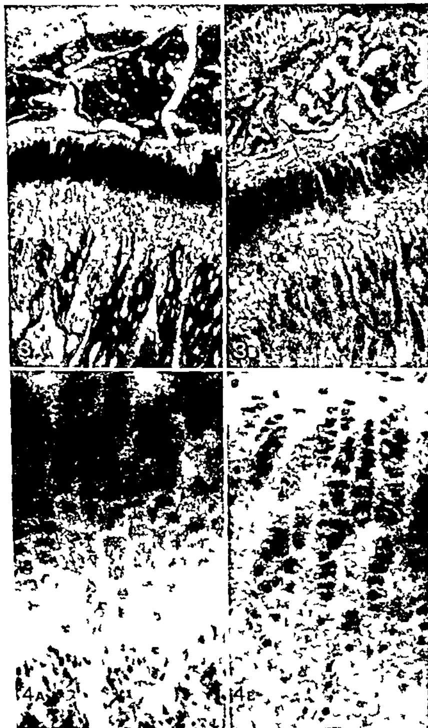
The cartilage generally showed less marked changes. Following administration of the large dose of actinomycin the final maturing and the disintegration of the chondrocytes did not seem to occur to a nor

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Figs 3–4

Fig 3a and b. Autoradiograms with ^{35}S of normal mouse (3a) and mouse treated with actinomycin as in group II (3b). There is a clear difference in the labelling of the chondrocytes. Magnification × 75. Haematoxylin.

Fig 4a and b. Autoradiograms with ^{35}S of normal mouse (4a) and mouse treated with actinomycin as in group II (4b). The same as in Fig 3 in greater magnification. × 160. Haematoxylin.


3A
3B
4A
4B

mal extent while the cells in the upper layer continued to proliferate at a normal rate. This resulted in unusually long columns of hypertrophic cells with dark nuclei.

A definite effect of actinomycin was its ability to retard but not to stop the incorporation of sulphur in cartilage, an effect seen already one day after administration of the small dose. This is in agreement with the observation made by *Salmon et al.* (1967). They showed that both puromycin and actinomycin have an inhibitory effect on sulphate incorporation by cartilage *in vitro*. This can be explained by the assumption that chondroitin sulphuric acid is linked to a protein component and that actinomycin and puromycin prevents formation of this protein.

Both in the actinomycin treated and control animals the radioactivity was seen mainly intracellularly in the cells of the germinative and proliferative layers of the epiphyseal of plate. This was probably due to the fact that the animals were sacrificed already two hours after injection of radioisotope by which time only part of the latter had been transported out into the matrix. In experiments (not described here) where the mice were given the same dose of ^{35}S as in this experiment but sacrificed after 24 hours, the radioactivity was seen mostly in the matrix.

SUMMARY

Actinomycin D was studied for its effect on the epiphyseal plate and the metaphysis in mice *in vivo* histologically and autoradiographically. The administration of actinomycin D caused a marked reduction of the uptake of ^{35}S by the cartilage cells indicating impaired synthesis of chondroitin sulphuric acid. The number of osteoblasts and osteoclasts as well as of haematopoetic cells was markedly reduced. The cellular changes in the epiphyseal plate were confined to impaired disintegration with abnormally long columns of hypertrophic cells as a result.

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GROWTH KINETICS OF THE MOUSE EPIDERMIS AFTER A SINGLE APPLICATION OF 3,4 BENZOPYRENE, CROTON OIL, OR 1,2 BENZOPYRENE

By

KJELL ELGJO

Received 18 x 67

In some previous investigations the growth pattern of the epidermal cell population of the mouse skin was evaluated after a single application as well as after repeated applications of benzene cantharidin and 20 methylcholanthrene respectively Three growth parameters were followed the mitotic count the mitotic duration and the mitotic rate (for precise definitions of these parameters see *Elgjo* 1966) After a single application the most important difference between methylcholanthrene and the two non carcinogens was that the former induced a very long lasting prolongation of the mitotic duration The period of prolonged mitotic duration lasted much longer than the hyperplasia of the epidermis and it could not well be interpreted as being part of a regenerative reaction

In the present study another three chemical compounds were studied 3 4 benzopyrene which is a complete skin carcinogen extensively tested (*Shubik & Hartwell* 1957) croton oil which is widely used as a promotor but which is in itself a complete although weak skin carcinogen (for review see *Hieger* 1961) and 1 2 benzopyrene which has no carcinogenic properties (*Lacassagne et al* 1963)

MATERIALS AND METHOD

Hairless mice (hr/hr) were used in all experiments The histology of their skin and the growth of the epidermal cell population have been well examined (*Crew & Mirskaia* 1932 *David* 1932 *Iversen & Evensen* 1962 *Iversen & Iversen* 1967)

The compounds were dissolved in acetone and 0 05 ml of the various agents was applied to the interscapular area by means of a special syringe The further experimental procedures have been described in previous papers (*Elgjo* 1966 *Elgjo* 1968)

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The mitotic rate and the mitotic duration were evaluated by means of the Colcemid method. The mitotic rate was calculated by means of the equation $R = \frac{C}{t}$ where R is the average mitotic rate and C the number of arrested mitoses t hours after the injection. In the present study t = 4 hours. The mitotic duration was calculated by means of the equation $D = \frac{M}{R}$ where D is the mitotic duration, M the mitotic count without Colcemid and R the mitotic rate. The method has been thoroughly discussed elsewhere (*Figsti & Dustin* 1955, *Elgjo* 1966).

The normal mitotic count, rate and duration of the epidermal cells of this strain of hairless mice were evaluated in a previous study (*Elgjo* 1966) comprising 100 mice not injected with Colcemid and 100 mice injected with Colcemid. The results were as follows:

Mitotic count (per 6 mm interfollicular epidermis): 6.74 ± 1.29
Mitotic rate (per 6 mm interfollicular epidermis per hour): 6.36 ± 1.19
Mitotic duration (hours): 1.07 ± 0.016

EXPERIMENTS AND RESULTS

The animals were treated with a single application of 0.05 ml of 1 per cent 3,4-benzopyrene in acetone, 6 per cent croton oil in acetone or 1 per cent 1,2-benzopyrene in acetone. Groups of 8 mice were sacrificed 1, 2, 4, 7 and 14 days after the application.

TABLE 1

Single Application of 0.05 ml of 1 Per Cent 3,4-Benzopyrene in Acetone

Time after application	Mean mitotic count without Colcemid	SE of the mean	Mean mitotic count after Colcemid	SE of the mean	Mitotic rate	Mitotic duration
1 day	6.50	1.84	22.00	1.15	5.50	1.18
2 days	12.75	2.87	31.75	3.28	7.94	1.61
4 days	12.75	1.32	71.25	9.82	17.81	0.72
7 days	15.50	2.18	42.25	2.66	10.56	1.47
14 days	7.75	0.50	36.50	6.65	9.13	0.84

TABLE 2

Single Application of 0.05 ml of 6 Per Cent Croton Oil in Acetone

Time after application	Mean mitotic count without Colcemid	SE of the mean	Mean mitotic count after Colcemid	SE of the mean	Mitotic rate	Mitotic duration
1 day	14.25	5.02	117.25	26.71	29.31	0.49
2 days	25.25	2.14	167.50	24.13	41.88	0.60
4 days	10.50	2.04	74.00	14.31	18.50	0.57
7 days	13.75	1.39	51.25	10.59	12.81	1.07
14 days	9.25	2.02	27.25	3.09	6.81	1.36

TABLE 3
Single Application of 0.05 ml of 1 Per Cent 1 2 Benzopyrene in Acetone

Time after application	Mean mitotic count without Colcemid	SE of the mean	Mean mitotic count after Colcemid	SE of the mean	Mitotic rate	Mitotic duration
1 day	8.25	1.17	67.00	10.81	16.75	0.49
2 days	9.75	0.72	33.00	10.33	8.25	1.18
4 days	5.25	1.38	33.75	4.40	8.44	0.62
7 days	9.25	0.85	35.75	4.30	8.94	1.03
14 days	11.25	3.42	18.50	3.66	4.62	2.43

The results are shown in Tables 1, 2 and 3, and in Figs. 1, 2 and 3. *After application of any of the 3 agents the mitotic rate increased* rapidly, attaining a maximum value 4 days after the application of 3,4-benzopyrene, 2 days after treatment with croton oil, and 1 day after application of 1,2-benzopyrene.

After application of 3,4-benzopyrene the mitotic duration was prolonged for the greater part of the experimental period. In contrast to this croton oil and 1,2-benzopyrene induced a reduction of the mitotic *duration during the first week. But during the 2nd week the mitotic duration increased to values above the normal range even after appli*cation of these two agents. The mitotic count varied considerably in all three experiments. These alterations were partly due to the alterations in the mitotic rate, partly to those in the mitotic duration.

DISCUSSION

In previous experiments (*Elgjo* 1966, *Elgjo* 1968) it was found that the initial reactions of the epidermal cell population to a single application of benzene, cantharidin or methylcholanthrene were well compatible with a simple regenerative reaction, the initial rise of the mitotic rate simply being the response to the cell loss induced by application of the agents (for detailed discussion of this see *Iversen & Evensen* 1962, *Iversen & Bjerknes* 1963 and *Skjæggestad* 1964). In the present experiments, too, the alterations of the mitotic rate and mitotic duration were highly indicative of a similar regenerative reaction. *Principally* the reaction appeared to be the same after application of any of the 3 agents. Some differences *should however be pointed out*.

After application of 1,2-benzopyrene the initial rise of the mitotic rate attained a maximum value already 1 day after the treatment. It is even possible that the rate had its maximum still earlier, as is the case with benzene (*Elgjo* 1966). This is in contrast to the more protracted reaction observed after application of croton oil or 3,4-benzopyrene. Here the highest mitotic rates were found on the 2nd and on the 4th

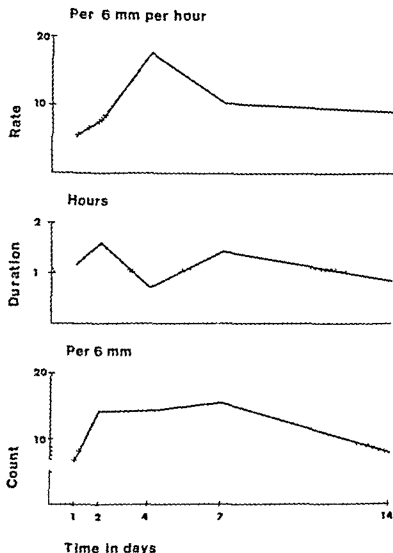


Fig 1

The mitotic rate, duration and count the first 14 days after application of 0.05 ml of 1 per cent 3,4 benzopyrene in acetone

days respectively. This protracted reaction following application of either of the two carcinogens was quite similar to the protracted reaction observed after application of methylcholanthrene (*Elgjo* 1966, *Elgjo* 1968). The cause of this slow reaction is not clear. It might be related to the degree of cell loss provoked by the various agents (*Skjæggestad* 1964), or to the mode and the degree of penetration into the skin. It might also be related to some disturbed metabolism in the epidermal cells, as it has been shown that hydrocarbon carcinogens accumulate and probably disturb the function of several important organelles in the cell, such as the mitochondria (*Iversen & Evensen*

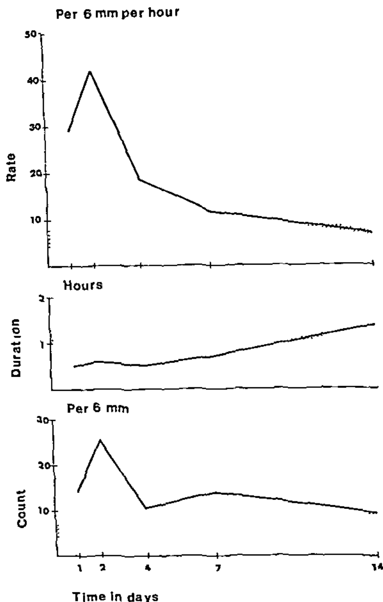


Fig 2

The mitotic rate durat on and count the first 14 days after application of 0 05 ml of 6 per cent croton oil in acetone

1962 *Bernhard & Tournier* 1966) the lysosomes (*Allison & Mallucci* 1964 *Shamberger & Rudolph* 1967) and even the nucleus (*Amlacher & Graffi* 1966)

In the present experiments the mitotic duration was consistently decreased during the initial period of rapid cell proliferation Thus no

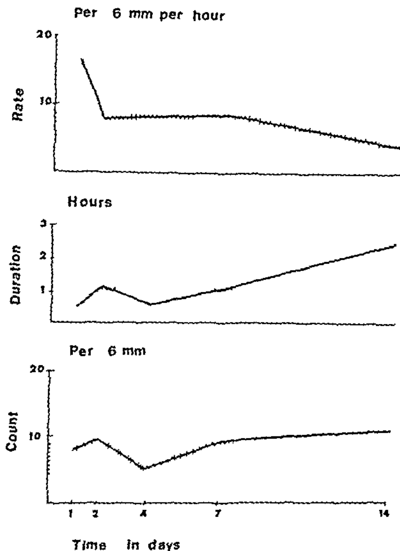


Fig 3

The mitotic rate duration and count the first 14 days after application of 0.05 ml of 1 per cent 1 2 benzopyrene in acetone

difference between the two carcinogens and 1 2 benzopyrene was found in this respect In fact the shortest mitotic durations were observed after application of croton oil This is in contrast to the findings after application of methylcholanthrene After treatment with this agent the mitotic duration tended to be longer than normal during all phases of the regenerative reaction even when the mitotic rate was high (Elgjo 1966 Elgjo 1968)

During the second week after the treatment the mitotic duration was prolonged after application of any of the three agents As indicated earlier (Elgjo 1966) this period of increased mitotic duration coincides

with the time during which the epidermis is hyperplastic As it is now fairly well established that the mitotic rate of the basal cells of the epidermis is regulated by an inhibitor produced by the differentiating cells (for review of this see *Bullough et al* 1967) and as it has been demonstrated that high concentrations of this inhibiting substance can prolong the mitotic duration (*Bullough & Laurence* 1964) it is quite possible that the long mitotic duration found during the period of epidermal hyperplasia can be related to an increased concentration of mitosis inhibiting substance (chalone) produced by the increased number of differentiating cells

As mentioned above the mitotic rate of the basal cell of the epidermis is probably governed by a mitosis inhibiting substance (chalone) produced by the differentiating cells When the growth of a cell population in this way is regulated by a negative feedback mechanism it can be expected to display some of the features common to all cybernetic systems (*Goldman* 1960 *Iversen & Bjerknes* 1963) When for instance the equilibrium of such a system is disturbed it can react with various types of oscillations (*Tustin* 1955) Thus the alterations in the mitotic rate induced by any of the 3 agents examined in this study can be looked upon as short lived damped oscillations in a cybernetic system In this respect the early reactions induced by three compounds examined in the present paper are quite similar to those provoked by benzene cantharidin and methylcholanthrene (*Elgjo* 1966 Elgjo 1968) It remains to be seen however if 3 4 benzopyrene and croton oil like methylcholanthrene can induce more long lasting alterations of the kinetics of the epidermal cell population

SUMMARY

The growth pattern of the epidermal cell population of hairless mice (hr/hr) was followed over a period of two weeks after application of 3 4 benzopyrene croton oil and 1 2 benzopyrene respectively The growth parameters were evaluated by means of the Colcemid method All three compounds induced alterations in the kinetics of the epidermal cell population that were compatible with simple regenerative phenomena The initial reaction however occurred somewhat later and was more sustained after application of the carcinogens (3 4 benzopyrene and croton oil) than after treatment with 1 2 benzopyrene Some possible explanations for this are discussed

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INTRASPINAL ENTEROGENOUS CYST

By

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Received 31 x 67

Intraspinal cysts lined by a mucosa of gastrointestinal type have by some been regarded as teratomas and by others as malformations The latter concept seems especially applicable to a group of 6 reported cysts called enterogenous cysts These appear to form a group in many respects different from the teratomatous cysts The purpose of this paper is to review the points of difference between the two groups of cysts as illustrated by a case of our own

CASE REPORT

A boy operated upon as a neonate for a thoracal myelomeningocele At the operation a thin walled cyst was found at the level of the myelomeningocele and located ventrally to the cord It was said to be removed Postoperatively he had normal tonus and motor activities of lower extremities However hydrocephalus soon developed and a shunt operation was performed. He thereafter developed signs of meningitis and succumbed 2 and a half months old

At autopsy (O 610/61 Lund) a meningitis was verified Ventrally to the cord lay an intraspinal extramedullary cystic tumour (Fig 1) only part of which apparently had been removed at the operation The tumour measured 20 × 35 mms and was surrounded by thickened meninges Histologically the cyst wall had a lining mucosa of intestinal type and consisted of connective and smooth muscle tissue forming laminae and coats as in a gut wall (Fig 2) A minor portion of the mucosal epithelium was columnar and ciliated and transgressed into squamous epithelium of oesophageal type No other types of tissue such as fat bone cartilage or skin appendages were present Adjacent portions of the cord showed an haemorrhage

The brain which was hydrocephalic showed multiple pea sized nodules of grey tissue bulging into the ventricular system (Figs 3 and 4) These nodules consisted of nerve cells scattered in a cortical type of neurophil These nodules were not continuous with the basal ganglia or the cortex Corresponding cortical areas showed micropolygyria (Fig 4) Sex chromatin staining according to *Klinger et al* (1957) showed no sex chromatin bodies in the mucosal epithelium of the intraspinal cyst

DISCUSSION

Intraspinal cystic tumours have been reviewed by *Hoefnagel et al* (1962) and *Rewcastle et al* (1964) In most of these cases the cyst was located dorsally to an intact cord only four being situated ventrally Vertebral closure defects were restricted to four cases with dorsal spina bifida none of which had a ventrally located cyst The cysts occurred in the lumbar as well as the thoracic and cervical region The cyst wall was usually thin and composed of fibrovascular tissue lined

by a simple layer of cylindrical or cuboid to squamous epithelium In some cysts the wall in addition showed fat bone cartilage striated or smooth muscle tissue and neural tissue A regularly built up gastro intestinal wall however was not seen except in the case of *Puusepp* (1934) here included among the enterogenous cysts These cysts have been regarded as teratomas a view supported by sex chromatin studies revealing in some cases cells of a sex different from that of the patient (*Hoefnagel et al* 1962 *Rewcastle et al* 1964)

There are however six reports on intraspinal cysts called enterogenous cysts (*Puusepp* 1934 *Guillery* 1937 *Korff* 1937 *Knight* 1955 *Rhaney et al* 1959 *Nemeth* 1965) These differed from the above described cysts in the following respects they had a wall completely replicating a gastrointestinal wall with an intestinal type of mucosa submucosa and layers of smooth muscle non intestinal tissue components were not seen they were all thoracocervical except for one dorsally located cyst (*Puusepp* 1934) they were intramedullar or lay ventrally to the cord or ventrally to and in between separated cord halves They were all associated with vertebral anomalies fusion of the vertebrae in one case dorsal spina bifida in two cases and ventral closure defects in three cases in one of the latter the intraspinal cyst communicated with a mediastinal enterogenous cyst and in one case a mesenteric tissue strand connected the cyst with the abdominal cavity

On the basis of the above listed differences these cysts could be separated from the teratomatous cysts The cyst described by *Puusepp* (1934) however was by *Hoefnagel et al* (1962) and *Rewcastle et al* (1964) included among the teratomatous although it seems to belong to the enterogenous cysts where it was also placed by *Harriman* (1958) in his review on enterogenous cysts Harriman on the other hand added a case of his own which judging from the description belongs to the teratomatous group

The enterogenous cysts are regarded as malformations deriving from endodermal primitive cells early in development possibly from cells detached from endoderm owing to faulty separation between endoderm and notochord or neuroectoderm (*Rhaney et al* 1959) or they may have emerged from a neurenteric duct (*Harriman* 1958) in connection with defect closure of the blastopore (*Cameron* 1957)

Figs 1–4

Fig 1 Transverse cut of spinal cord (top left) malformed and split up by an haemorrhage Ventrally to this (below) lies the cystic tumour lined by mucosa × 5 v Gieson

Fig 2 The wall of the cyst lined by a mucous membrane layered loose connective tissue and smooth muscle tissue × 100 v Gieson

Fig 3 Frontal lobes viewed from behind with heterotopias bulging into the widened ventricular system.

Fig 4 Frontal cortex showing micropolygyria and separated from the cortex heterotopic grey tissue at ventricular lining 5 Htx eosin

Ventral vertebral closure defects were not observed at the autopsy of our case but a small defect may have been overlooked or may have been closed by fibrous tissue. No spinal X-ray studies were made. The absence of sex chromatin in the epithelial cells of the cyst speaks against though it does not exclude the possibility of a teratoma.

The present cyst perfectly replicating a mature gastrointestinal wall devoid of non-intestinal tissue lacks conclusive features of a teratoma. It fits into the group of the 6 previously described enterogenous cysts and should be regarded as a malformation here combined with Arnold-Chiari's malformation. It is noteworthy that *Cameron* (1957) described an abdominal enterogenous cyst and *Rhaney et al.* two enterogenous cysts (1959) one of which was intraspinal and one mediastinal all occurring in cases of Arnold-Chiari's malformation. We also want to stress that in this case there were CNS malformations not belonging to the Arnold-Chiari's syndrome proper. This concurrence though described by a few authors does not seem to be widely recognized. It may be of importance for the discussion of the pathogenesis of the Arnold-Chiari's malformation as well as from a therapeutic surgical and prognostic point of view.

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DIFFERENT TYPES OF ANTIBODIES WITH A GLIOTOXIC EFFECT IN SERUM FROM ANIMALS WITH EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS

By

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Received 31.x.67

Experimental allergic encephalomyelitis (EAE) is thought to be an auto-immune disease of the central nervous system. When inducing it with a homogenate of whole brain or spinal cord tissue mixed with Freund's complete adjuvant the production of antibodies to at least some of the antigenic determinants in the homogenate must be expected. With the aid of ordinary immunological methods such antibodies have also been demonstrated, for example to central nervous tissue as such by immune precipitation in agar gel and immunoelectrophoresis (*Berg & Dencker* 1962), to an ethanolic extract of brain tissue by the complement fixation test (*Paterson* 1959, *Paterson et al.* 1965), to cerebroside by complement fixation and precipitation (*Niedieck et al.* 1965) or to basic encephalitogenic protein by haemagglutination or gel diffusion (*Alvord et al.* 1965). When the disease is induced with a water soluble encephalitogenic protein circulating antibodies to the antigen can be demonstrated with I^{131} labelled encephalitogen (*Kibler & Barnes* 1962) or by a modified tanned cell method (*Caspary & Field* 1965).

The tissue culture technique offers another method in the investigation of humoral factors at EAE. A factor that effects a demyelination of cultures containing myelinated fibres and also produces cell death among cultured glia cells has been demonstrated in serum from EAE animals sensitized with a homogenate of brain or spinal cord and Freund's adjuvant (*Bornstein & Appel* 1961, *Lamoureux et al.* 1966). The myelinotoxic activity was dependent on complement and was entirely in the γ (7S) globulin fraction of DEAE column eluents (*Appel & Bornstein* 1964, *Bornstein & Appel* 1965). *Berg & Källén* (1962) have described a similar gliotoxic effect of EAE serum on young

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brain cultures Further interest in these factors was evoked by the observations that the same cytotoxic effects were obtained with sera from patients with neurological diseases among these multiple sclerosis (*Berg & Kallén* 1961 1962*b* *Bornstein* 1962)

The present paper is concerned with the pattern of gliotoxic factors in serum from animals sensitized with encephalitogenic antigens of varying purity Three kinds of encephalitogenic materials have been used alternately in order to induce EAE homogenized spinal cord tissue encephalitogenic protein and a pH 9 dialysate of such a protein The ability of the two last mentioned antigens to inhibit the gliotoxic activity of different serum fractions *in vitro* has also been examined

MATERIALS AND METHODS

Animals Randomly bred rabbits of both sexes (weighing about 2–2¼ kg) were used throughout the investigation

Induction of EAE The animals were injected in three foot pads with a mixture containing equal parts of encephalitogenic material and Freund s complete adjuvant (Difco) When lyophilized encephalitogenic antigen was used it was dissolved in distilled water before being mixed with the adjuvant The type and the total amount of antigen which each animal received are shown in Tables 1–3

Encephalitogenic antigens Bovine spinal cord tissue served as the homogenized brain antigen The encephalitogenic proteins used were two preparations from bovine brain Preparation A was made according to the method described by *Kies* (1965) The modified acid extraction procedure proposed by Kies was followed The four acid extracts were combined proteins were precipitated with ammonium sulphate and the encephalitogenic material was purified with DEAE cellulose and lyophilized Preparation B was made in a somewhat different way Homogenization lyophilization and defatting of the tissue were carried out according to *Lumsden et al* (1966) except that the petroleum spirit extraction was omitted Then the encephalitogenic protein was prepared as follows The partially defatted powder was extracted with 0 1 M Na acetate pH 5 made 4 per cent in regard to NaCl and with 10 per cent NaCl acidified with HCl to pH 2 These two extracts were combined and dialyzed against distilled water pH was adjusted to 6 with NaOH and the solution was treated with ammonium sulphate to 80 per cent saturation The precipitate was dissolved in a minimum of water and dialyzed against a large amount of distilled water for three days at 4 C The suspension in the bag was filtered and the clear solution was chromatographed on DEAE cellulose according to *Kies* (196) The material eluted with water was collected and lyophilized This material constitutes preparation B of encephalitogenic protein An aliquot of preparation B was further treated by dialysis in water made pH 9 with NaOH according to *Lumsden et al* (1966) The outside water was collected twice during two months and lyophilized The recovered material was fractionated by Sephadex C 100 gel filtration in phenol acetic acid water (1 1 1) and two fractions were collected and lyophilized (Fig 1) The fractions were solubilized in distilled water and freed from phenol by Sephadex G 25 gel filtration and lyophilized This material constitutes fractions I and II of alkaline dialysate of encephalitogenic protein referred to in the following as fractions B I and B II

Sera At various times after the challenge injections (in most cases every seventh day) blood samples (approximately 10 ml) were collected from the rabbits The serum was separated by centrifugation as soon as coagulation had taken place and was immeditely frozen The sera were stored in the deepfreeze (—20 C) until used

DEAE Sephadex Chromatography The DEAE Sephadex separations were performed as follows The serum sample (3–4 ml) was thawed and dialyzed in the refrigerator against 0 03 M NaCl 0 075 M tris HCl pH 8 for 2–3 days It was then put on a column of DEAE Sephadex A 50 (bed dimensions approximately 1 2 × 20 cm) which was carefully equilibrated with the above mentioned buffer The sample was washed down into the gel bed with a few ml of the buffer and the

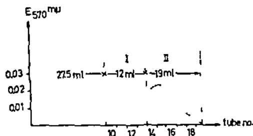


Fig 1

Sephadex G 100 gel filtration of an alkaline dialysate of encephalitogenic protein (preparation B) Elution buffer phenol acetic acid water (1 1 1) Effective bed dimensons 1 4 × 4? cm Sample volume 3 ml Flow rate 9 ml/hour

proteins were eluted with the same buffer with a linearly increasing concentration of NaCl Finally when most of the proteins were eluted the molarity of NaCl in the eluent was stepwise increased to ? M and a last fraction was collected The UV absorption at ?54 mµ was continuously recorded by an Uvicord and the eluate was automatically collected In the first part of the investigation (rabbits Nos 200 ?03 and 213–?19) the tubes from the fraction collector were pooled to ? fractions (Fig ?) At the other separations (concerning sera from rabbits Nos 2?5 231) fraction 5 was further divided into fraction 5a and 5b (Fig 3) The fractions were concentrated by ultrafiltration the buffer was changed to saline by Sephadex G 2 gel filtration concentration was again performed this time to half the volume of the original serum sample Finally the fractions were filtered through a Millipore filter (0 22 µ) The whole fractionation procedure was carried out during 4 5 days and in the cold (+4–8 C)

Sephadex G–?00 Gel Filtration These separations were performed according to *Flodin & Killander* (196?) The gel bed had the effective dimensions of approximately 2 5 × 90 cm and was cooled by running tap water The elution was carried out with 0 4 M NaCl 0 1 M tris HCl pH 8 The UV absorption at ?54 mµ was monitored by a Uvicord and the eluate automatically collected The tubes from the fraction collector corresponding to the three protein peaks were pooled but in most cases fraction II was subdivided into fractions II_a and II_b (Fig 4) The pooled fractions were further treated as described above for the DEAE Sephadex fractions before being tested for gliotoxic activity Sometimes 0 02 ml of a 10 per cent solution of dextran sulphate 2000 and 0 1 ml of 1 M CaCl was added to each ml of the serum sample The mixture was centrifuged after 15 minutes and the supernatant was then gel filtered This procedure is said to deplete the serum of β_2 lipoproteins

Treatment of gliotoxic fractions with mercaptoethanol An aliquot of the concentrated gliotoxic fraction (in tris HCl buffer) was mixed volume by volume with 0 2 M ? mercaptoethanol The mixture was held at 4 C for ?4 hours then Sephadex G 25 filtered in saline the protein fraction was concentrated to the appropriate volume and Millipore filtered

Test of gliotoxic activity The gliotoxic activity of the fraction was tested on glia cell cultures from neonatal rat which were made with reconstituted rat tail collagen as previously described (*Berg & Kallen* 19?3) The cultures were used as test object – seven to ten days old— when a mat of neuroglia cell had developed The fluid medium as carefully poured off the cult a rin e l with nutrient solution (Parker 199) and 0 5 ml of the rabbit s r m f acti n w a added After incubating the culture for approximately 30 minut t 37 C the serum fraction was poured off The culture was rinsed with Parker 199 and a mixture of 0 9 ml Parker 199 and 0 1 ml of fresh normal guinea pig r m w a ded The culture was examined after the incubation with the eru fr t n n l e ers thirt min utes after the addition of guinea pig serum To t th l t xic effe t a s ring of 0 + or ++ was used

0 No or onl slight degeneration of the g l ltre l ith n t o hours.
\+ A moderate degenerative change of m l ia cell c l oe l served

after one hour and a rather strong reduction in their number occurred within two hours

++ A clearly visible degeneration of most of the cells had taken place after 30 minutes and a complete dissolution of most glia cells was observed within one to two hours

Inhibition of gliotoxic activity with encephalitogens The possibility of inhibiting the activity of a gliotoxic serum fraction with encephalitogenic antigen was examined as follows 0 5 ml of the fraction was mixed with 0 5 ml of a solution of encephalitogen in distilled water In most case the concentration of encephalitogen in the mixture was 300 μg/ml 100 μg/ml or 25 μg/ml The mixture was incubated for 30 minutes at 37 C and then tested for gliotoxic activity as described for the serum fractions Simultaneously the gliotoxic activity of an untreated portion of the serum fraction was checked In the same way tests were made with incubated mixtures of the basic protein protamin sulphate and gliotoxic fraction The protamin sulphate was a commercial preparation from herring (L Light and Co Ltd Colnbrook England) The protein was solubilized in Parker 199 before being mixed with the fraction Its final concentration in the mixture was 100 μg/ml It was also examined to see whether the solutions of encephalitogen and protamin sulphate (together with guinea pig serum) had any noxious effect on the glia cells

RESULTS

DEAE Sephadex Separations

All serum samples collected were fractionated by DEAE Sephadex chromatography The UV absorption pattern at 254 mμ from the different separations showed a good resemblance (Figs 2 and 3) Each fraction of all sera was tested for gliotoxic activity

Six normal rabbit sera were investigated Four were samples taken from each of the rabbits Nos 216–219 just before they were given the sensitizing injections None of these four (or two other normal sera not included in Tables 1 3) showed gliotoxic activity in any one of its fractions

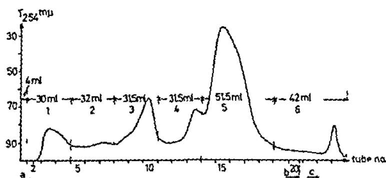


Fig 2

DEAE Sephadex A 50 chromatography of a serum obtained from rabbit No 201 21 days after challenge Effective bed dimensions 1 9 × 9 cm Sample volume 4 ml Elution buffer a) 4 ml (the sample) followed by 5 ml 0 03 M NaCl 0 075 M tris HCl pH 8 b) A gradient of linearly increasing NaCl molarity in 0 075 M tris HCl pH 8 buffer was applied by connecting a beaker with 150 ml of 0 4 M NaCl 0 075 M tris HCl pH 8 with an identical mixing beaker containing 150 ml of the starting buffer Elution was performed with 170 ml of this gradient system c) A stepwise change to 2 M NaCl 0 0 5 M tris HCl pH 8 Flow rate approximately 10 ml/hour

TABLE 1

The Pattern of Ch t xic Factors in Serum from Rabbits Sensiti ed with 0 1o ml of a Mixture of Homogeni ed Bovine Spinal Cord and Freun l s Complete Adjuvant (1 1) after Fractionation with DEAE Seph idex (Fig 9)

Rabbit	No 200						No 201						No 202						No 203					
Clinical signs	Positive day 13						Positive day 13						Negative						Negative					
Hi tolog signs	Positive						Positive						Negative						Positive					
Fraction	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
um ul																								
11)	++	++	0	+	++	0	+	0	0	++	++	0	0	+	0	0	0	0	0	0	0	+	++	+
((	+	+	0	+	++	0	++	0	0	0	+	0	0	0	0	0	++	0	++	0	0	++	++	0
7) ,							++	0	0	0	++	0	0	0	0	0	0	0	++	0	0	0	++	0
40 da s		+	0	0	+	0	++	0	0	0	0	0	0	0	0	0	0	0	++	0	0	0	++	+
51 days							++	+	0	0	++	0												

TABLE

The Pattern of Gliotoxic Factors in Serum from Rabbits Sensitized with Encephalitogenic DF4F Sephadex

Rabbit	No 216						No 217					
Dose	150 µg of prep A						150 µg of prep A					
Clinical signs	Positive day 14						Positive day 11					
Histolog signs	Positive day 52						Negative day 52					
Fraction No	1	2	3	4	5	6	1	2	3	4	5	6
Serum sample												
0 days	0	0	0	0	0	0	0	0	0	0	0	0
12 days							0	0	0	++	+	0
17 days												
27 days							0	0	0	0	+	0
33 days	0	0	0	0	+	0						
4[illegible] days	0	0	0	0	+	0	0	0	0	0	++	0
52 days	0	0	0	0	++	0	0	+	0	0	+	0

Rabbit	No 214						No 215					
Dose	150 µg of prep A						150 µg of prep A					
Clinical signs	Positive day 11						Positive day 12					
Histolog signs	Positive						Positive					
Fraction No	1	2	3	4	5	6	1	2	3	4	5	6
Serum sample												
7 days												
14 days	0	0	0	0	+	0	0	0	0	0	+	0
21 days												

These rabbits were given a booster dose on day 44

Four rabbits (Nos 200–203) were challenged with whole bovine spinal cord tissue Three of them (Nos 200 201 and 203) developed EAE as judged from clinical and histological examinations Serum samples taken from these rabbits between the 14th and the 51st day post challenge showed a gliotoxic activity in fractions 1 and 5 except twice once only fraction 1 was active and once only fraction 5 The fourth rabbit (No 202) showed no clinical symptoms or histological signs of EAE This rabbit only twice showed a gliotoxic effect in its serum fractions (in one case the effect was slight)

Ten rabbits were challenged with basic encephalitogenic protein of preparation A (Nos 213 219) or B (Nos 229–231) All developed EAE within 21 days but none of them ever gave a gliotoxic reaction in serum fraction 1 Nine however were gliotoxic in serum fraction 4 and/or 5 in all samples tested The tenth (No 219) only once showed that effect

Protein of Preparation A or B in Freund's Complete Adjuvant after Fractionation with (Figs 2 and 3)

No 218						No 219						No 213					
150 μg of prep A						150 μg of prep A						150 μg of prep A					
Positive day 11						Positive day 16						Positive day 12					
Spontaneous death day 18						Negative						Not done					
1	2	3	4	5	6	1	2	3	4	5	6	1		3	4	5	6
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+	0
0	0	0	0	++	0	0	0	0	0	0	0						
						0	0	0	0	0	0						
						0	0	0	0	+	0						

No 229							No 230							No 231						
75 μg of prep B							75 μg of prep B							75 μg of prep B						
Positive day 16							Positive day 21							Negative day 14						
Positive							Positive							Positive						
1	2	3	4	5a	5b	6	1	2	3	4	5a	5b	6	1	2	3	4	5a	5b	6
0	0	0	0	++	+	0	0	0	0	0	+	++	+	0	0	0	0	0	0	0
0	0	0	0	+	+	0	0	0	0	0	++	++	0	0	0	0	++	0	0	0
0	0	0	0	0	+	0	0	0	0	+	++	++	+							

Four rabbits (Nos 225–228) were sensitized with the alkaline dialysate of encephalitogenic protein (preparation B I and B II) All developed EAE within 21 days A gliotoxic effect in fraction 1 was never observed in sera from these animals but every one gave a gliotoxic serum fraction 4 and/or 5 at least in the 21 days sample

The results of the DEAE Sephadex separations are summarized in Tables 1–3

Sephadex G 200 Gel Filtrations

Ten Sephadex G 200 gel filtrations were performed with sera taken from rabbits Nos 225–231 21 days after challenge (Fig 4) Six serum samples were separated without prior treatment with dextran sulphate and four were separated after such treatment In four of the six gel

TABLE 3

The Pattern of Cytotoxic Factors in Serum from Rabbits Sensitized with an Alkaline Dialysate (Fraction B I or B II) of Encephalitogenic Protein of Preparation B in Freund's Complete Adjuvant after Fractionation with DEAE Sephadex (Fig 3)

Rabbit	No 225							No 226							No 227							No 228						
Dose	75 μg of B I							75 μg of B I							75 μg of B II							75 μg of B II						
Clinical signs	Positive day 17							Positive day 19							Positive day 21							Positive day 16						
Histol signs	Positive							Positive							Positive							Positive						
Fraction No	1	2	3	4	5a	5b	6	1	2	3	4	5a	5b	6	1	2	3	4	5a	5b	6	1	2	3	4	5a	5b	6
7 days	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	++	0
14 days	0	0	0	0	0	0	0	0	0	0	0	++	0	0	0	0	0	0	0	+	0	0	0	0	0	0	++	+
21 days	0	0	0	0	++	++	0	0	0	0	0	0	++	0	0	0	0	++	0	++	0	0	0	0	0	0	++	0
	0	0	0	++	++	0	0																					

This serum was fractionated twice giving almost identical elution patterns at 254 mμ

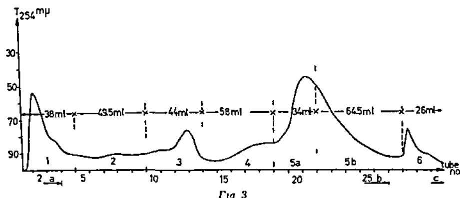


Fig 3

DEAE Sephadex A 50 chromatography of a serum obtained from rabbit No 227 21 days after challenge Effective bed dimensions 1.0 × 19 cm Sample volume 3 ml Elution buffer a) 3 ml (the sample) followed by 30 ml 0.03 M NaCl-0.075 M tris HCl pH 8 b) A gradient of linearly increasing NaCl molarity in 0.075 M tris HCl pH 8 buffer was applied by connecting a beaker with 200 ml of 0.3 M NaCl-0.075 M tris HCl pH 8 with an identical mixing beaker containing 200 ml of the starting buffer Elution was performed with 257 ml of this gradient system c) A stepwise change to 2 M NaCl-0.075 M tris HCl pH 8 Flow rate approximately 10 ml/hour

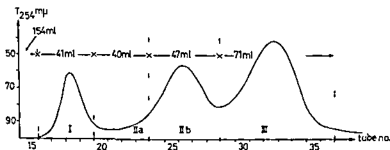


Fig 4

Sephadex G 200 gel filtration of a serum obtained from rabbit No 227 21 days after challenge Effective gel bed dimensions 2.5 × 90 cm Sample volume 3 ml Elution buffer 0.4 M NaCl-0.1 M tris HCl pH 8 Flow rate approximately 10 ml/hour

filtration experiments with untreated sera fraction IIb was gliotoxic and in two cases a gliotoxic effect at the same time could be demonstrated in fraction IIa whereas fractions I and III were inactive in these four cases In the other two experiments with untreated sera gliotoxic activity was restricted to fraction I However when the separations of these two last mentioned sera were repeated with dextran sulphate treated samples the activity was now found in fractions IIa and IIb whereas fraction I was inactive The remaining two dextran sulphate treated sera showed gliotoxic activity only in fraction IIb The cause of the different localization of the gliotoxic factor in untreated and dextran sulphate treated samples of the same sera which was

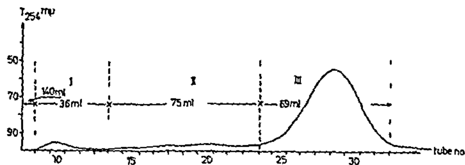


Fig 5

Sephadex G 200 gel filtration of DEAE Sephadex fraction 5 obtained from the serum of rabbit No 201 21 days after challenge Effective gel bed dimensions 2.5 x 89 cm Sample volume 1.5 ml Elution buffer isotonic NaCl Flow rate approximately 7 ml/hour

observed in two cases could be an interaction between the gliotoxic factor and lipoproteins during the gel filtration of the untreated sample

Twice a gliotoxic fraction 5 from the DEAE Sephadex separations was further fractionated on Sephadex G 200 (Fig 5) In both cases the gliotoxic effect could be demonstrated only in fraction II

The results of these experiments are summarized in Table 4

Treatment of gliotoxic fractions with 2 mercaptoethanol

The gliotoxic activity of DEAE Sephadex fraction 1 was unaffected in both of the samples investigated whereas gliotoxic fractions 4 and 5 were completely inactivated in all six examined specimens The treatment caused also Sephadex G 200 fractions II_a and II_b to lose their cytolytic effect

The results are summarized in Table 5

TABLE 4

The Pattern of Gliotoxic Factors in Serum from Rabbits with EAE after Separation by Sephadex G 200 Gel Filtration (Fig 4)

Sample	I	II IIa	 IIb	III
Fraction 5 (No 201 21 days)	0	++		0
Fraction 5 (No 201 27 days)*	0	+		0
No 225 21 days	0	+	++	0
No 225 21 days D S †	0	0	++	+
No 226 21 days	+	0	0	0
No 226 21 days D S †	0	+	+	
No 227 21 days	0	++		0
No 228 21 days	0	0	+	0
No 229 21 days	+	0	0	0
No 229 21 days D S †	0	+	++	
No 230 21 days D S †	0	0	++	
No 231 14 days	0	0	++	0

* On this occasion the elution was performed with saline

† D S indicates that this serum sample was treated with dextran sulphate before being separated

TABLE 5

Activity of Gliotoxic Fractions from FAF Sera Tested Before and after Treatment with 2 Mercaptoethanol

Sample	Before	After
Fraction 1 (No 203 27 days)	++	++
Fraction 1 (No 203 40 days)	++	+
Fraction 5 (No 203 27 days)	++	0
Fraction 5 (No 203 40 days)	++	0
Fraction 4 (No 2?7 ?1 days)	++	0
Fraction 5b (No 227 21 days)	++	0
Fraction IIa (No 2?7 21 days)	++	0
Fraction 4 (No 231 14 days)	++	0
Fraction IIb (No 231 21 days)	++	0
Fraction 5b (No 2?5 21 days)	++	0

Tests of untreated and treated aliquots of the fractions were performed at the same time except for fraction 5 (No ?03 27 days) and fraction 4 (No ?31 14 days) which were examined 1? days before mercaptoethanol treated specimens

Inhibition of Gliotoxic Activity with Encephalitogens

Attempts were made to inhibit the activity of gliotoxic fractions by incubation with encephalitogenic proteins prior to test It was found that the gliotoxic effects of DEAE Sephadex fractions 4 or 5 were strongly inhibited by preparation B B I or B II at concentrations of these antigens from 300 to 25 µg/ml of the antigen gliotoxic fraction mixture Incubation of these fractions with protamin sulphate did not affect the activity Efforts to inhibit the gliotoxic activity of fraction 1 with encephalitogenic protein (preparation A) failed

The results of the inhibition studies are summarized in Table 6

DISCUSSION

A gliotoxic effects in serum from animals with LAE has previously been demonstrated by *Berg & Kallen* (1962) These investigations were extended here to an examination of the gliotoxic effect of different serum fractions from rabbits sensitized to develop FAF with different encephalitogenic materials

Complement Dependence of the Gliotoxic Factors

Preliminary investigations showed that all of the fractions from DEAE Sephadex separations of FAF sera were gliotoxically inactive when tested without any supplemental additions On the other hand a toxic effect could be demonstrated in some fractions if they were mixed with fresh normal guinea pig or human serum Moreover if such a fraction with strong gliotoxic a[illegible] ty was incubated for approximately 30 minutes at 37 with a gl[illegible] ll culture and the fraction then carefully poured off a complete ar[illegible] gliotoxic re[illegible] tion could

TABLE 6

Activity of Gliotoxic Fractions before and after Incubation with Encephalitogenic Antigens or Protamin Sulphate for 30 Minutes at 37° C

Sample	Gliotoxic activity of fraction only	Gliotoxic activity of encephalitogen (or protamin sulp.) and guinea pig serum	Gliotoxic activity of encephalitogen (or protamin sulphate) gliotoxic fraction mixture		
Fraction I (No 200, 21 days)	+		1500 A +		
Fraction I (No 201, 21 days)	++	25 A 0	25 A ++		
Fraction I (No 201 day 21 and 27 pooled)	+		900 A +		
Fraction I (No 201, 40 days)	++		250 A ++		
Fraction 4 (No 227, 21 days)	++	100 B II 0	100 B II ++		
Fraction 5b (No 227, 21 days)	++	100 B II 0	100 B II 0		
Fraction IIa (No 227, 21 days)	++	100 B II 0	100 B II 0		
Fraction 5a (No 227, 21 days)	++	300 B 0	300 B 0	100 B 0	25 B 0
Do	++	300 B I 0	300 B I +	100 B I 0	25 B I +
Do	++	300 B II 0	300 B II 0	100 B II 0	25 B II 0
Fraction 5a (No 229, 7 days)	++		100 B 0	100 B I 0	100 B II 0
Fraction 4 (No 231, 14 days)	++	100 P 0	100 B 0	100 P ++	
Fraction IIb (No 231, 14 days)	++		100 B 0		

Figures given indicate final concentration (in µg/ml) of encephalitogen in the encephalitogen gliotoxic fraction mixture or in the encephalitogen—guinea pig serum mixture tested. A indicates preparation A and B preparation B of encephalitogenic protein. B I and B II the two fractions of alkaline dialysate of protein B and P protamin sulphate.

be achieved by adding to the culture 0.1 ml of guinea pig serum mixed with 0.9 ml of Parker 199. Obviously the gliotoxic factor has an affinity to the glia cell. Its cytolytic activity depends on the presence of complement. These results confirm that the gliotoxic factor is of antibody nature (*Appel & Bornstein* 1964; *Bornstein & Appel* 1965; *Pette et al* 1965). *Berg & Kallen* suggested earlier that heating the serum to 56° C for 30 minutes inactivated the gliotoxic factor in EAE serum. The

gliotoxic effect was thought not to be restored by the addition of guinea pig serum this however is incorrect The gliotoxic effect demonstrated in EAE serum by *Berg & Källén* has later been shown to be reconstituted after heat inactivation by the addition of fresh normal guinea pig or human serum (*Berg & Källén* 1965)

Two Types of Gliotoxic Antibodies IgG and IgA

When EAE was induced in rabbits with a homogenate of bovine spinal cord tissue at least two types of gliotoxic antibodies separable by DEAE Sephadex chromatography could be demonstrated in their serum One type is eluted in the first fraction and the other in fraction 4 and/or 5 Sera from EAE animals sensitized with basic encephalitogenic protein or with an alkaline dialysate of such protein showed gliotoxic antibodies in fraction 4 and/or 5 (except for one rabbit No 219) but failed to show any gliotoxic effect in fraction 1 even after seven weeks Both types of gliotoxic antibodies when present seem to appear in the serum in a demonstrable amount during the second week after challenge and remain—possibly with a slight variation in intensity—for at least seven weeks

If rabbit serum is chromatographed on an anion exchanger such as DEAE cellulose or DEAE Sephadex the first protein fraction eluted mainly or exclusively consists of γG globulins As far as is known the cytological properties of antibodies of the IgG type are not affected by treatment with mercaptoethanol The activity of the gliotoxic factor in DEAE Sephadex fraction 1 is insensitive to such a treatment Thus there are strong reasons for considering this factor to be an antibody of the IgG type This is consistent with earlier findings that the demyelinating factor in EAE serum from rabbits sensitized with whole brain tissue is localized to the γ_2(7S) globulin fraction (*Appel & Bornstein* 1964 *Bornstein & Appel* 1965)

The gliotoxic factor in the DEAE Sephadex fractions 4 and/or 5 has not been previously demonstrated These fractions of the rabbit serum can be expected to contain the bulk of the γA and γM globulins Gel filtration on Sephadex G 200 separates serum proteins in respect to molecular shape and size giving three major fractions The γM globulins are eluted in the first peak and the γA and γG globulins in the second The third fraction mainly consists of albumin When separating serum that is gliotoxic only in DEAE Sephadex fraction 4 and/or 5 by this method the gliotoxic factor is eluted in the second fraction Gel filtration of isolated gliotoxic DEAE Sephadex fractions 4–5 gives the same results Thus there seems to be no evidence of a gliotoxic antibody of the IgM type Treatment with mercaptoethanol inactivates the gliotoxic antibody present in DEAE Sephadex fraction 4 and/or 5 The experiments rather indicate that this gliotoxic factor might be an antibody of a type similar to human IgA It is worth noticing that the

gliotoxic activity of this factor is dependent on complement Immunoglobulins of the IgA type have been recently demonstrated in the serum of rabbits (*Onoue et al* 1964 *Cebra & Robbins* 1966)

Unfortunately specific antisera to rabbit immunoglobulins have not been available to us during the investigation Therefore examination of the gliotoxic fractions using double diffusion in agar gel of immunoelectrophoresis has not been made

A Pathogenic Role for Gliotoxic Serum Antibodies?

EAE can be transferred passively with cell suspensions from lymph nodes of immunized animals How these cells act on the central nervous tissue of the host animal is not known The opinion is often expressed that the sensitized cells are capable of penetrating the blood brain barrier and of gathering around the myelin and glia cells where they could perhaps produce antibodies to these target cells (*Paterson* 1966) Efforts to transfer EAE with immune serum failed until *Jancovic et al* (1965) by local administration of antibrain serum into the lateral ventricle in normal animals succeeded in inducing histological lesions characteristic of EAE The factor in immune serum responsible for this transfer could be a demyelinating and gliotoxic antibody in which case and under certain circumstances (a defective blood brain barrier) it could play a pathogenic role In the present investigation two such gliotoxic antibodies have been demonstrated One tentatively identified as an IgA was present in the sera of 16 out of 17 EAE animals independent of the kind of encephalitogenic material used to induce the disease The other which is of the IgG type is not produced when the disease is induced with pure encephalitogenic material

It is not known whether the gliotoxic antibodies have a pathogenic role in EAE A recent investigation by *Winkler & Arnason* (1966) of experimental allergic neuritis (EAN) in rat is of great interest in this context They succeeded in inhibiting a cell mediated *in vitro* demyelination by preincubating the sensitized cells with rabbit antiserum to rat IgA This effect seemed to be specific to IgA antiserum since antisera to some other serum proteins including IgG did not affect the cytotoxic activity of the sensitized cells To throw light upon the question of a pathogenic role for different gliotoxic antibodies the *in vivo* method described by *Jancovic et al* (1965) could possibly be used

Inhibition of Gliotoxic Activity with Encephalitogenic Antigens in vitro

The gliotoxic activity of the IgA antibody is inhibited by incubating the fraction with encephalitogenic protein or an alkaline dialysate of the protein prior to test No such inhibiting effect could be achieved by incubating the gliotoxic antibody with the basic protein protamin sulphate Thus this inhibition seems to be based upon immunological specificity rather than upon a non specific affinity of the gliotoxic

factor for basic proteins A specific inhibition of the gliotoxic effect with encephalitogenic antigen would be evidence of a close relation (possibly identity) between an antigenic determinant on the glia cell and one of the encephalitogen However it would not prove that this determinant is identical with the encephalitogenic one

On the other hand the gliotoxic activity of the IgG antibody is not inhibited by incubation with encephalitogenic protein This is consistent with the fact that it is absent from EAE sera taken from animals sensitized with the protein and indicates that it is evoked by and directed against an antigen in the spinal cord homogenate different from the encephalitogenic protein

If the described inhibition test is based on immunological specificity it can be used in further attempts to examine the specificity of gliotoxic antibodies of different origin including human for encephalitogenic antigens The technique might also prove valuable in investigations of the antigenic determinant of encephalitogenic materials

SUMMARY

Experimental allergic encephalomyelitis (EAE) was induced in rabbits with a homogenate of bovine spinal cord tissue with basic encephalitogenic protein or with an alkaline dialysate of such protein Serum from the rabbits was fractionated by DEAE Sephadex chromatography and Sephadex G 200 gel filtration and the fractions were tested for gliotoxic activity Two types of gliotoxic antibodies were demonstrated one is of the IgG type and the other which has not previously been described shows some properties which would permit to classify it as an IgA Its gliotoxic action though is dependent on the presence of complement Both kinds of gliotoxic antibodies are present in sera from rabbits sensitized with whole spinal cord tissue Rabbits immunized with purified encephalitogen show gliotoxic antibodies only of the IgA type The question of a pathogenic role for the gliotoxic serum antibodies is discussed

Incubation of the gliotoxic IgA fraction with encephalitogenic protein prior to test inhibits the gliotoxic activity Such an inhibition with encephalitogenic protein is not valid for the gliotoxic IgG antibody The described inhibition technique would probably be valuable in examinations of the immunological specificity of different gliotoxic antibodies and encephalitogenic antigens

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INVESTIGATIONS ON THE ENZYMES AND TOXINS OF STAPHYLOCOCCI

Study of Hyaluronidase by the Viscosimetric Method

By

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Received 30 x 67

The occurrence of a spreading factor in the cultures of staphylococci which was correlated to their invasiveness in the skin of rabbits was demonstrated by *Duran Reynals* (6) The identity of the spreading factor with the mucolytic enzyme hyaluronidase was established by *Chain & Duthie* (5) These studies stimulated further research on the possible role hyaluronidase played in staphylococcal infections Results of these investigations outlined by *Elek* (7) indicated that the production of hyaluronidase by staphylococci was as common as their ability to form coagulase and that the virulent organisms shared the property of producing a number of components of which one was hyaluronidase

Although no definite conclusion may be drawn as yet as to the relationship of hyaluronidase to the virulence of staphylococci it is recognized that the enzyme may promote the spreading of other infections *Lack* (16) demonstrated a synergistic action of vaccinia virus and staphylococci which correlated closely with the presence of hyaluronidase produced by the bacteria *Packalen* (22) and *Bergqvist* (2 3) suggested that hyaluronidase of staphylococci and other bacteria may aid in the dissemination of tuberculosis These results indicate that hyaluronidase might be of importance for the effectiveness of staphylococcal vaccines Experiments were therefore made to establish the optimal conditions for the preparation purification and properties of the enzyme

MATERIALS AND METHODS

Strains and culture supernatants 400 clinical strains were grown in Difco brain heart infusion broth for 5 days at 37 C and the supernatants were prepared a outlined earlier (27)

Production of hyaluronidase Staph aureus Wall r a laboratory strain is among the high hyaluronidase producers This strain s therefore used for the preparation of the enzyme The bacteria were grown for three days at 37 C in a casein hydrolysate medium under conditions im l t a haemolysin p paration (26) A gas mixture consisting of 25 per cent carbon di xide and 75 p r ent air was passed over the surface at the rate of appr xim tel 00 ml per m nut After growth the contents of different flasks were poo'e l ntrifuged f 0 m utes at

2500 g at 4 C and the supernatant was Seitz filtered Sodium merthiolate was added to the filtrate to a concentration of 0 01 per cent The filtrate was concentrated 30 times by ultrafiltration at 4 C using Berkefeld filters coated with 10 per cent collodion and centrifuged at 30 000 g for 30 minutes at 4 C. The clear supernatant was used in the chromatographic studies as the source of the enzyme

Spectrophotometric determinations The absorbance at 280 mμ of the fractions from chromatographic experiments was measured using a Beckman spectrophotometer model DU with 1 cm light path cells

EXPERIMENTAL

A number of methods are in use for the determination of hyaluronidase Among these is the viscosimetric method which has a high degree of reproducibility (15 24) This method was therefore used in the present study although the performance of the tests were tedious and time consuming

The viscosimetric method was studied earlier by *Hultin* (9 12) and applied for the assay of amylase (19) proteolytic activity (13 14 18 28) lysozyme (19, 20) as well as hyaluronidase (12 13) Preliminary studies were done to standardize the method for the determination of the staphylococcal enzyme

Relationship of Concentration of Hyaluronic Acid to Viscosity

Potassium hyaluronate (Sigma) from human umbilical cord was used as the substrate The rate of flow at 25° C of solutions of different hyaluronic acid concentration in 0 1 M citrate buffer of pH 4 8 through an Ostwald viscosimeter was studied 0 4 per cent hyaluronic acid gave a flow time of 171 6 seconds and the flow time decreased proportionally with decrease in hyaluronic acid concentration, yielding a time of 17 5 seconds for the buffer alone (Fig 1) A concentration of 0 15 per cent hyaluronic acid gave a flow time (τ) of 46 seconds This amount was used in the subsequent studies as it was desirable to make the measurements repeatedly at intervals of one minute

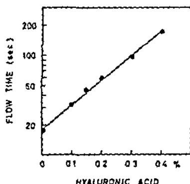


Fig 1

Relationship between the concentration of hyaluronic acid and the flow time through an Ostwald viscosimeter

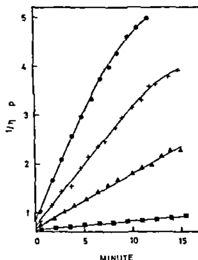


Fig 2

The rate of reduction in viscosity of hyaluronic acid by different concentrations of staphylococcal hyaluronidase

(●——● 684 H U ×——× 390 H U ▲——▲ 166 H U ■——■ 27 H U)

Effect of Enzyme Concentration on Rate of Reduction of Viscosity

The reaction mixture for studies on the determination of the enzyme activity consisted of 1 50 ml of 0 4 per cent hyaluronic acid 2 4 ml of 0 1 M citrate buffer of pH 4 8 and 0 1 ml of the culture filtrate containing the enzyme adjusted to pH 4 8 The reduction in viscosity was followed by repeated measurements of the flow times (τ) and the reciprocal of specific viscosity (ηsp) was calculated according to the formula as described below

$$\frac{\tau_{sample} - \tau_{buffer}}{\tau_{buffer}} = 1/\eta sp$$

The factor $1/\eta$sp increased proportionally with decreasing viscosity brought about by the action of the enzyme on hyaluronic acid The effect of different amounts of an enzyme preparation in brain heart infusion broth on the change of $1/\eta$sp values at various times is shown in Fig 2 Reaction curves typical of enzyme activity were obtained In order to be able to compare the activity of one preparation against another the formula described by *Hultin* (15) and *Lundblad* (17 18) was applied The Hultin Units (HU) so obtained indicated the activity (A) in terms of viscosity reducing units per ml of enzyme per second for the quantity of substrate concentration used

$$A = \frac{a+b}{a} \; C \; \frac{2}{s} \; \frac{d \frac{1}{\eta sp}}{d\tau} = HU \times 10^{9}$$

Where a = gram (ml) enzyme solution
b = gram (ml) substrate solution
C_S = the concentration of the substrate in the reaction mixture (gram substrate per gram solution)
ηsp = specific viscosity (ηrel-1)
t = time in seconds

For the convenience of calculation in routine analysis of culture supernatants, a standard curve was drawn relating the initial reaction velocity obtained (1/ηsp per minute) with activity in Hultin Units (Fig 3)

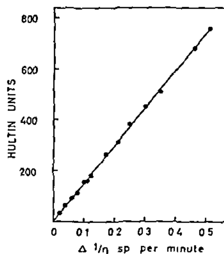


Fig 3

The relationship between the rate of reduction in viscosity and the activity of hyaluronidase expressed in Hultin units

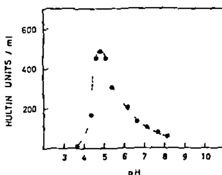


Fig 4

Staphylococcal hyaluronidase pH activity relationship

Relationship of pH to Enzyme Activity

The relationship of pH to enzyme activity as well as the viscosity of hyaluronic acid was studied using 0 15 per cent hyaluronic acid with

different buffer systems No activity was demonstrable below pH 3 6 and above 8 5 As the pH was increased above pH 3 6 the activity increased rather sharply giving a maximum at pH 4 8 Further increase in pH reduced the enzyme activity (Fig 4) The staphylococcal hyaluronidase was thus found to have a pH optimum at 4 8

The viscosity of hyaluronic acid at various pH values was measured using the same viscosimeter The viscosity was not affected between pH 5 and 10 as a flow time of 45 seconds was obtained in this range Below pH 5 and above 10 the flow time decreased somewhat At the pH values of 3 4 and 11 the flow times were 39 43 and 41 seconds respectively

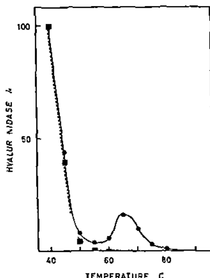


Fig 5

Thermostability of hyaluronidase ●——● crude enzyme (1320 H U /ml) and ■——■ purified enzyme (1550 H U /ml)

Temperature Stability of Hyaluronidase

Aliquots of the culture filtrate containing 1320 Hultin Units per ml were exposed to different temperatures for 30 minutes in a constant temperature water bath (± 0 1 C) cooled to room temperature and stored overnight at 4 C Activity measurements done at 25 C and at pH 4 8 showed that the enzyme was rapidly inactivated as the temperature was raised from 40 to 50 C (Fig 5) Eight per cent of the activity remained after exposure to 50 C for 30 minutes However when the enzyme was exposed to temperatures of 60 and above a certain increase in the activity was noticed 15 per cent of the initial activity was demonstrable after heating at 65 C Further increase of temperature decreased the activity of the enzyme No activity was demonstrable after exposure to 80 C or higher temperature

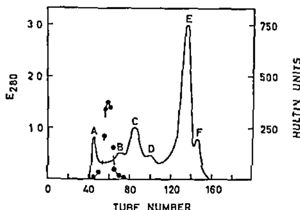


Fig 6
Purification of hyaluronidase by gel filtration on Sephadex G 100

Similar experiments were performed with the enzyme purified by chromatography on Sephadex G 100 as described below The enzyme had an activity of 1550 Hultin Units per ml Activity decreased with progressive increase in temperature as with the crude enzyme and the activity was not demonstrable after exposure to 55 C for 30 minutes However further increase in temperature did not show any reactivation It was probable that the activity with unfractionated enzyme obtained after heating to 65 C was due to reactivation by some component on cooling similar to that noticed with testicular hyaluronidase (24) This component was apparently removed during chromatography

Purification of Hyaluronidase by Gel Filtration on Sephadex G 100

10 ml of the concentrated culture filtrate was applied on a column of Sephadex G 100 (Pharmacia Uppsala) with a gel bed size of 2 cm dia meter and 140 cm length using 0 05 I TRIS HCl buffer of pH 7 6 at 4 C 3 ml portions of the effluent were collected in tubes in an auto matic fraction collector Measurement of the absorbance at 280 mμ of the fractions gave the distribution pattern outlined in Fig 6 The pres ence of six fractions (A B C D E and F) was observed

Hyaluronidase activity determinations on the fractions showed that the enzyme was located in the fractions coming after the first peak A and before the appearance of the second peak B Peak A represented the void of the gel and contained substances of molecular weights of 100 000 or more representing the exclusion limit for the gel Tubes 58 and 59 contained hyaluronidase in maximal amounts and from its position a molecular weight of 80 000–85 000 could be assigned to hyaluronidase A total of 19 200 Hultin Units was applied on the col umn and 21 720 units were recovered Thus no activity was lost during the fractionation

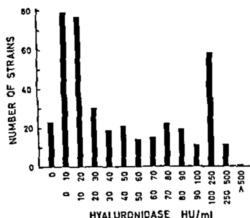


Fig 7
Production of hyaluronidase by clinical strains

Production of Hyaluronidase by Clinical Strains

The occurrence of hyaluronidase in the supernatants of 400 clinical strains was studied 23 strains (5 75 per cent) did not produce any activity 226 strains (56 50 per cent) liberated hyaluronidase less than 50 HU/ml and 151 strains (37 75 per cent) produced larger than 50 HU/ml (Fig 7) Thus a larger number of strains were producers of low amounts of hyaluronidase

DISCUSSION

Methods for the determination of hyaluronidase (8 12 21 24) are based on the splitting of the larger molecule of hyaluronic acid into smaller fragments with either lower viscosity decreased reactivity with basic proteins or inability to form mucin clot Although reproducible none of these procedures is ideal or correct from a strictly theoretical consideration of enzyme kinetics as compared with the study of other methods of enzyme assay For example the viscosity reduction method does not distinguish between the viscosity of the initial larger split products and one obtains only a mean of the values In the turbidity reduction method the turbidity formation is not lost until the split products attain molecular weights of lower than 6 000–8 000 (21) The initial molecular size of the substrate is therefore of importance Further the turbidity of the particles can differ in size and shape affecting the spectrophotometric determinations The viscosimetric method was chosen in the present study as it was shown to have a high degree of reproducibility (24)

Experiments by different workers have given different pH optima for staphylococcal hyaluronidase Thus *Bergquist* (4) found an optimum of between pH 5 5 and 6 0 whereas *Rogers* (23) described an op

timum around pH 6 6 In the present study a pH value of 4 8 was found to be optimal and the pH activity curve was well defined pH optima are no criteria for the characterization of enzymes and this is especially true in the case of the phosphatases (25) Such optima are known to be influenced by ionic strength concentration of enzyme presence of inhibitor concentration and molecular size of the substrate as well as the type of buffer solutions used It is also possible that the enzyme produced by various strains have different pH optima

The effect of different temperatures on the stability of the crude enzyme showed that there was a slight reactivation at 60–70 C although the major portion of the activity disappeared after exposure to 55 C for 30 minutes No such reactivation was noticed with the purified enzyme A similar reactivation on cooling after the heat treatment was demonstrated with testicular hyaluronidase (24)

Gel filtration studies showed that staphylococcal hyaluronidase had a molecular weight corresponding to between 80 000 and 85 000 The chromatographic pattern and the location of hyaluronidase was similar to the preparation from the S6 strain reported by *Hallander* (9) It was also reported that staphylococci produced multiple molecular size forms of hyaluronidase (1) The occurrence of a sharp peak of enzyme activity on the chromatograms noted in the present study indicated that a single size was probably involved The increase in activity after gel filtration may be due to the removal of some inhibitory component

SUMMARY

The viscosimetrical method for the determination of staphylococcal hyaluronidase was studied and found to be convenient for routine use The enzyme had a pH optimum at 4 8 and 95 per cent of its activity was lost on exposure to 55 C for 30 minutes However a demonstrable reactivation occurred in the crude unfractionated enzyme between 60 and 70 C Gel filtration chromatography on Sephadex G 100 showed that the enzyme occurred in the inner volume and from its position on the chromatogram a molecular weight of between 80 000 and 85 000 was assumed

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IMMUNOLOGICAL RELATIONSHIPS OF *STREPTOCOCCUS MG* TO GROUP K STREPTOCOCCUS, STRAIN K 4a

By

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Received 5 ix 67

The capsular antigen characteristic of streptococci designated by *Mirick et al* in 1944 as *Streptococcus MG (Strep MG)* was found to be related to but not identical with the capsular antigen of *Streptococcus salivarius (Strep saliv) type I* (8 9 10)

Later *Farmer* (2) demonstrated antigens common to *Strep saliv* and streptococci of *Lancefield* s group K a finding which was confirmed by *Williams* (16)

In 1964 *Willers et al* (15) published data which indicated the identity of carbohydrate antigens of *Strep MG* and the type III antigen of group F streptococci and described the serological relationships between these streptococci and *Strep saliv* type I (8606 NCTC[1])

During routine diagnostic work with streptococci from human blood cultures a number of non haemolytic streptococci (n h strep) were encountered the extracts of which strongly precipitated both antisera against *Strep MG* and antisera against a non haemolytic non levan producing strain of group K K 4a (NCTC) *(Strep K 4a)*

Further investigations of this serological relationship were prompted by current studies on the possible relation of *Strep MG* to primary atypical pneumonia (p a p)

MATERIAL AND METHODS

Strains

The following reference strains of n h strep were used *Strep MG* strain 8037 (NCTC) = Ottens group F III and strain B (own collection) Strep of group K strain K 4a (NCTC) *Strep saliv* type I strain 8606 (NCTC) type II strain Packham (NCTC) Strep of group F strain Is 57 (15) Strep of group K train Perrot (Wellcome Lab CN 477) and strain Levy (NCTC) Strep of group H strain Challis (own collection) Strep of group M strain 1085 (Wellcome Lab) Strep of group C strain Niel (4) Strep of group L strain D 167 A (ATCC)

Eighteen strains identified as *Strep MG* (from 13 cases) and 14 strains identified

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1 National Collection of Type Cultures Colindale England
American Type Culture Collection Rockville Maryland

as *Strep K 4a* (from 9 cases) were isolated from blood of patients suffering from various diseases e g subacute bacterial endocarditis pneumonia and pleuritis One strain representing each case was subjected to the complete panel of investigations described below The investigations were later extended to encompass a further 6 strains of *Strep MG* and 9 strains of *Strep K 4a* isolated from patients to be described in the following article (6)

Antisera

Rabbits were immunized by repeated intravenous injections of vaccine until an agglutinin titre of ≥ 320 with the homologous antigen was attained The vaccines were prepared from cultures of bacteria grown for 18 hours in trypsin digested broth (12) or Todd Hewitt broth (9) The cultures were killed by heat (65 C for 60 min) or by 1 per cent formalin washed three times and suspended in saline with 0 5 per cent formalin in a dense suspension Vaccines of a strain prepared by means of the two killing procedures produced antisera with roughly the same reactions

Bacterial extracts for immunoprecipitation were prepared according to the methods described by *Lancefield* (5) and *Fuller* (3) at 120 C

Identification and Classification of the Strains Isolated

This was performed by the following procedures

1 *Morphology* of the colonies was observed on horse blood agar The cells were studied in a Gram stained smear of an 18 hour culture from trypsin digested broth after washing in saline
2 *Haemolysis* was demonstrated around colonies grown on 5 per cent horse blood agar
3 *Immunoprecipitations* were performed in microtubes with a diameter of 1 5–2 mm or by a microtechnique of double diffusion in agar gel (14) in the latter case the precipitation lines were recorded daily by drawings and on the third day by drying the preparations and staining with Amidoschwartz B or by photography
4 *Agglutination* in tubes was read after 20 hours in a water bath at 50 C
5 *Quellung reactions* by Neufeld s method were carried out using prevaccination and postvaccination rabbit sera and methylene blue
6 For *absorptions* one third to one fifth wet volume of packed cells was added to undiluted serum and allowed to react for 1–2 hours at 37 C in some cases followed by further reaction at 4 C overnight when necessary the procedures were repeated
7 The *indirect fluorescent antibody technique* was applied as described elsewhere (7)

The strains were examined by the following *biochemical and cultural procedures*

8 Growth on horse blood agar containing 40 per cent ox bile
9 Production of NH_3 from L arginine HCl demonstrated by Nessler s reagent.
10 Hydrolysis of sodium hippurate shown by the addition of 50 per cent H_2SO_4 to two parts of the supernatant broth culture
11 Production of levan on 5 per cent sucrose agar
12 Hydrolysis of esculin and starch using Difco s broth base
13 Production of acid during growth for 8 days in broth containing glucose sucrose lactose maltose salicin trehalose raffinose arabinose mannitol inulin sorbitol and glycerol using Difco s broth base and phenol red as indicator
14 Sensitivity to sulphathiazol and the antibiotics penicillin streptomycin chloramphenicol and tetracycline was measured as described by *Thomsen* ((13) p 111)

RESULTS

Morphological and Cultural Characteristics

The *Strep MG* and *Strep K 4a* strains isolated were small Gram positive cocci growing in short chains or pairs On 5 per cent horse blood agar the *Strep MG* strains formed small whitish colonies of vary

ing size within the same culture some strains produced a faint α haemolysis while the others did not produce haemolysis The *Strep K 4a* strains formed small uniform grey or whitish colonies In some strains and most obviously in the reference strain of *Strep K 4a* a dissociation into small whitish glossy colonies (white form) and bigger greyish colonies (grey form) took place The former always dissociated The latter was stable during numerous passages The two forms were indistinguishable by the immunological and biochemical tests employed In fluid medium the grey form of *Strep K 4a* multiplied as a homogeneous suspension, while the white form rapidly sedimented with a turbid supernatant All strains and forms of *Strep K 4a* were α haemolytic

Immunological Reactions

The fact that strain K 4a belongs to Lancefield's group K was checked by cross precipitation in microtubes with two reference group K strains and their homologous antisera Extracts of *Strep MG* occasionally weakly precipitated antisera against group K (Table 1)

TABLE 1

Cross Precipitation of Streptococcus K 4a and MG and Two Strains of Group K Streptococci with Their Homologous Antisera

Extracts of	Antisera against Group K strain Levy	Group K strain Perrot	Strep K 4a	Strep MG
Group K strain Levy	+++	++	+++	—
Group K strain Perrot	++	+++	++	—
Strain K 4a	+++	++	+++	+++
Strep MG strain 8037	— or +	— or +	+++	+++

Precipitation in microtubes Reactions within 5 min = +++ between 5 and 10 min = ++ and between 10 and 15 min = + No precipitation = —

Extracts of the *Strep K 4a* reference strain were not precipitated by antisera against groups M and F whereas a weak precipitation line occurred with an antiserum to group H by double diffusion in gel

The *Strep K 4a* strains isolated were agglutinated to almost the same titre as were the *Strep MG* isolates in antisera against both strains both showed the characteristic disk like agglutination in the lower concentrations of antisera Analogous strong reactions were recorded in cross precipitation tests By precipitation in gel Lancefield and Fuller extracts of *Strep MG* and *K 4a* reacted with both these antisera forming lines which indicated a reaction of identity (Fig 1) In some experiments other lines indicated the presence of non related antigens in these microorganisms

Quellung occurred for all *Strep K 4a* and *MG* strains in both antisera the Quellung of *Strep K 4a* was however not as distinct as that of *Strep MG*

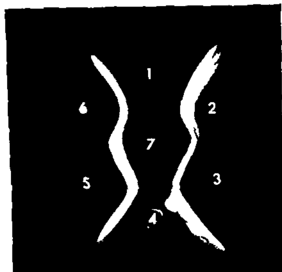


Fig 1

Precipitation by double diffusion in agar gel
2 = Fuller and 3 = Lancefield extracts of Strep MG 5 = Fuller and 6 = Lancefield extracts of Strep K 4a 1 and 4 = anti Strep MG rabbit serum
7 = anti Strep K 4a rabbit serum

Cross absorptions of rabbit antisera carried out with the reference strains of *Strep K 4a* and *Strep MG* revealed differences in their antigenic structure as indicated in Table 2 *Strep K 4a* absorbed all agglutinins and precipitins from *Strep MG* antiserum whereas *Strep MG* was not able to remove all agglutinins and precipitins from *Strep K 4a* antiserum the antibody residue did not react with extracts of the group K strains Levy and Perrot

Other absorption experiments were performed with serum from a patient with p a p which in addition to antibodies against *Mycoplasma pneumoniae* contained antibodies against *Strep MG* as demonstrated both by agglutination and indirect fluorescent antibody technique This

TABLE 2

Immunological Cross Reactions with Streptococcus K 4a *and* Streptococcus MG

Antigen	Antisera from rabbits vaccinated with												Pre acc serum
	Strep K 4a			Strep MG			I 4 ab w th MC			MC abs with K 4a			
	Q	A	P	Q	A	P	Q	A	P	Q	A	P	A
Strep K 4a	+	320	+++	+	640	++-		3	-	—	<10	—	<2
Strep MC	+	320	+++	+	640	+++		10	—	—	<10	—	<2

Q = Quellung reaction A = titre l tul t P = precipitat on in microtubes within 5 min = +++ l t 10 and 15 min —

serum agglutinated *Strep MG* and *Strep K 4a* and also *Strep saliv* type I to almost the same titre These titres were strongly reduced by cross absorptions but not by the immunologically distinct *Strep saliv* type II (Table 3)

The presence of antigens of Lancefield s groups C F and L (15 11) was found in a proportion of the *Strep K 4a* and *Strep MG* strains isolated The results are given in Table 4

TABLE 3

Immunological Cross Reactions with Streptococcus K 4a Streptococcus MG *and* Streptococcus salivarius *Type I*

Agglutination with	Titres of human serum absorbed with				
	Nil	K 4a	MG	saliv type I	saliv type II§
Strep K 4a	45	<16	<16	<16	36
Strep MG	90	<16	<16	<16	72
Strep saliv type I	60	<16	<16	<16	70
Strep saliv type II	<16	<16	<16	<16	<16
FAT with Strep MG	240	<20	<20	<20	160

From patient with primary atypical pneumonia § Immunologically non related to *Strep MG* FAT = Fluorescent Antibody Test

TABLE 4

Serological Relation of Streptococcus K 4a *and* Streptococcus MG *to Lancefield s Groups C F and L*

Extracts of	Number of strains	Number of strains precipitating antisera against group		
		C	F	L
Strep MG	20	7	9	1
Strep K 4a	19	(2)	0	4

Brackets = weak reaction

Biochemical Characteristics

The biochemical reactions characterizing the strains of *Strep MG* and *K 4a* isolated are contained in Table 5 compare *Mirick et al* (8 9 10) This table also applies to the classification of n h strep described in the following article (6)

From Table 5 it appears that fermentation of esculin trehalose and raffinose together with growth on 40 per cent ox bile agar offer means of distinction with a high degree of certainty between the two serologically related n h strep *Strep MG* and *Strep K 4a* All strains of *Strep MG* grew well on the 40 per cent ox bile agar whereas this property was only present in 47 per cent of the *Strep MG* strains investigated by *Mirick et al* (8) Three of the 15 *Strep MG* strains failed to

hydrolyse esculin and two did not split trehalose The uncertainty due to these missing reactions is greatly reduced by the criterial demand (Table 5 foot notes) that if a n h strep which in other respects reacts as a *Strep MG* 1) does not grow well on 40 per cent ox bile agar either esculin or trehalose must be fermented 2) if it does not ferment esculin then trehalose must be fermented and 3) if it does not ferment trehalose then esculin must be fermented

TABLE 5

Criteria for the Classification of Streptococcus MG *and* Streptococcus K 4a

Tests	Strep MG	Strep K 4a	Reactions used as criteria for Strep MG	Reactions used as criteria for Strep K 4a
Agglutination and Quellung reaction by anti Strep MG	15/15	10/10	+	+
Precipit in gel of HCl and Fuller extr by anti MG and anti K 4a with reaction of identity	15/15	10/10	+	+
α haemolysis on blood agar	11/15	10/10	no or faint	+
Full growth on 40 per cent ox bile blood agar	15/15	0/10	+	— α
NH_3 from arginine	15/15	10/10	+	+
Hydrolysis of hippurate	0/15	0/10	—	—
Production of levan	0/15	0/10	—	—
Fermentation of esculin	12/15	0/10	+ β	—
starch	0/15	0/10	—	—
glucose, sucrose, lactose, maltose	15/15	10/10	+	+
salicine	15/15	3/10	+	+ or —
trehalose	13/15	0/10	+ γ	—
raffinose	0/15	9/10	—	+ or — μ
arabinose, mannitol, inulin, sorbitol, glycerol	0/15	0/10	—	—
Resist to sulphathiazol	15/15	8/10	+	+ or —
Sensitivity to penicillin 4 U per ml	15/15	?(8) 10	+	+ or —

Number of strains isolated from blood inclusive f rence strain reacting ut of t tal If negati e either esculine or trehalose mu t b fermented , n i γ If negati e growth on ox bile agar must be demanded α If positive and μ if negative then neither esculin nor trehal mu t be fermented

One of the 10 strains of *Strep* K 4a failed to ferment raffinose The growth of all 10 strains was either completely or partly inhibited on the ox bile agar If a n h strep which in other respects reacts as a *Strep* K 4a does not ferment raffinose or shows full growth on ox bile agar then the criterial demand is set up that neither esculin nor trehalose must be fermented (Table 5 foot notes)

As a supplement to these criteria it is noted that 2 out of 10 *Strep* K 4a strains were sensitive to sulphathiazol (238 μg per disk) whereas the other K 4a and all 15 *Strep* MG strains were resistant to this drug Furthermore 8 strains (one of them being identical with a sulphathiazol sensitive one) of *Strep* K 4a were only relatively sensitive to penicillin (4 units per disk) while the other 2 K 4a and all 15 *Strep* MG strains were sensitive to this antibiotic All *Strep* MG and K 4a strains were sensitive to chloramphenicol and tetracycline most of the strains were relatively sensitive to streptomycin

Nine *Strep* K 4a strains isolated from p a p patients (6) were tested for sensitivity to penicillin (4 units per disk) 6 were weakly sensitive 2 were relatively sensitive and one was sensitive the latter was also sensitive to sulphathiazol while the other 8 were resistant All 6 *Strep* MG strains from the same isolation trial were sensitive to penicillin and resistant to sulphathiazol

DISCUSSION

Non haemolytic streptococci is a well established term for streptococci which do not produce a soluble haemolysin However until recent studies by *Colman* and *Williams* (cf 1) no satisfactory classification of this group of bacteria had been attempted

The present article describes a number of n h strep isolated from blood they were naturally grouped together with one of two reference strains which apart from their common immunological reactions behaved differently in a series of biochemical reactions On the basis of the morphological cultural immunological and biochemical characteristics criteria were set up for n h strep which may be classified as *Strep* K 4a and as *Strep* MG in accordance with the criteria laid down for *Strep* MG by *Mirick et al* (8 9 10)

During the present study many n h strep were recorded which by biochemical and cultural behaviour were similar to *Strep* K 4a and *Strep* MG and whose extracts moreover precipitated antisera to these two organisms with varying but generally lower strength However the lack of reaction of identity by precipitation in agar between the latter and the n h strep in question together with great discrepancies in titres of agglutination by *Strep* K 4a and MG antisera led to their exclusion from this study

The inability of *Strep* MG completely to remove all agglutinins from *Strep* K 4a rabbit antiserum (Table 2) can hardly be compared to its

reduction of antibodies in the human p a p serum which reacted with *Strep K 4a* (Table 3) The antibodies in the former were elicited by vaccination of rabbits and reacted to a much higher titre than those of the latter serum which were presumably produced during stimulation by the related antigens of *Mycoplasma pneumoniae* causing the in fection

Mirick et al (8) showed that the capsule of *Strep MG* contained a polysaccharide which is responsible for its type specific serological reactions *e g* agglutination and precipitation The reactions of *Strep MG* and *K 4a* both in homologous and heterologous antisera indicate that the related antigens may be confined to the capsule of these orga nisms as visualized by the Quellung reaction

No attempts were made to correlate the occurrence of *Strep MG* or *K 4a* to the disease of the patients from whose blood they were isolated The relation of *Strep MG* and *Strep K 4a* to p a p is discussed in the following article (6)

The demonstration that a proportion of the strains reacted with antisera to Lancefield s group C F and L is in agreement with other reports (15 11) Preliminary absorption and precipitation experiments have indicated however that the reactions of *Strep MG* in antisera to group C and group L depend on partially related antigens since potent precipitins to group C and group L respectively were left in the ab sorbed sera Absorption and precipitation experiments with group F antisera gave inconclusive results for *Strep MG* containing the group F antigen The solution of these serological problems must await further elucidation

SUMMARY

A number of non haemolytic streptococci isolated from the blood of patients with various diseases were classified as either *Streptococcus MG* (*Mirick et al* 1944) or *Streptococcus K 4a* They were immunologic ally closely related but behaved differently in a series of biochemical reactions

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ISOLATION OF NON HAEMOLYTIC STREPTOCOCCI FROM PATIENTS WITH PRIMARY ATYPICAL PNEUMONIA

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In the early 1940s the aetiology of primary atypical pneumonia (p a p) was the object of extensive studies These led to the demonstration in 1944 by *Eaton et al* (10) of an agent which was later identified as a mycoplasma (2) designated *Mycoplasma pneumoniae (M pneum)* (3) this organism was proved to be of aetiological significance in a proportion of patients with p a p and milder respiratory infections (15 16 6 1)

At the same time a non haemolytic streptococcus (n h strep) designated *Streptococcus MG (Strep MG)* was studied in relation to the aetiology of p a p *Strep MG* was isolated in 1943 by *Thomas et al* (22) from the lungs of a patient who died of p a p Development of agglutinins against this streptococcus was demonstrated in a significantly higher proportion of sera from patients with p a p than of sera from patients with other conditions or from healthy individuals (23 11) *Strep MG* agglutinins have been reported to develop with varying but low frequencies in patients with infections due to *M pneum* (8 16 6 4 21 7 12) *Strep MG* was isolated more frequently from patients with p a p than from healthy persons and patients with other respiratory infections (23) This finding has not been confirmed by later investigations (5 21)

The aim of the present investigations was to elucidate the possible significance of *Strep MG* in p a p In association with a *M pneum* isolation study (13) attempts were made to isolate n h strep from patients with p a p Special attention was paid to *Strep MG* and the immunologically related *Streptococcus K 4a (Strep K 4a)* (20) and to the occurrence of antibodies against *Strep MG* The results were evaluated in relation to disease isolation of *M pneum* and antibodies against *M pneum*

MATERIAL AND METHODS

Patients

Attempts to isolate n h strep were made in the last 62 of the 77 patients previously described in the *M pneum* isolation study (13) According to the clinical diagnosis 52 of these patients suffered from pneumonia including 30 with p a p or virus pneumonia three had respiratory infection without pneumonia and seven had no respiratory infection

Identification and Classification of N H Strep

Throat swabs were spread immediately on a semiselective medium containing gentian violet 1 500 000 sodium azide 1 50 000 and sulphapyridine 1 2000 as described by *Thomas et al* (23)

N h strep were identified by the following criteria

1) Formation of small greyish or white colonies on the semiselective medium More than 10 identical colonies present
2) Gram positive cocci growing in short chains or pairs as demonstrated by microscopy of an 18 hour culture from trypsin digested broth
3) α haemolysis or no haemolysis on agar containing 5 per cent horse blood

No attempts were made to quantify the number of colonies on primary culture

The strains identified as n h strep were classified as described in the preceding article (20) *Streptococcus salivarius* type I which is immunologically related to *Strep MG* was classified by the method reported by *Mirick et al* (19)

The strains were divided into three groups i e 1) *Strep MG* 2) *Strep K 4a* and 3) the remainder

Serological Methods

The *Strep MG* agglutinin test was performed according to the method of *Thomas et al* (23) modified only with regard to titration as described previously (13)

The techniques of the indirect fluorescent antibody test (FAT) for titration of *M pneum* antibodies and the cold haemagglutinin test have also been described (13)

RESULTS

Isolation of n h strep was attempted in 62 patients isolation was successful in 42 of these resulting in a total of 49 strains 7 of the patients accounting for two strains each (Table 1)

Thirty of the patients suffered from primary atypical or virus pneumonia Eighteen strains of n h strep were isolated from 16 of these patients *Strep MG* and *Strep K 4a* were represented by two and three strains respectively The remaining 32 patients had other respiratory infections or no respiratory infection N h strep were isolated from 26 of these resulting in 31 strains including four strains of *Strep MG* and six strains of *Strep K 4a* (Table 1) The results in the group of patients with p a p or virus pneumonia are not significantly different from those in the group of patients with other or absence of respiratory infections

Streptococcus salivarius was not isolated in this study

The correlation between the isolations of *Strep MG* and *Strep K 4a* and the demonstration of a) *Strep MG* agglutinins and b) antibodies against *M pneum* is shown in Table 2 All six strains of *Strep MG* originated from patients presenting a negative test for *Strep MG*

TABLE 1

Non Haemolytic Streptococci Isolated from 62 Patients

Age group	Primary atypical or virus pneumonia					Other or no respiratory infections				
	Patients		Strains			Patients		Strains		
	Isolation		Non haemolytic strep			Isolation		Non haemolytic strep		
	attempted	succeeded	MG	K 4a	other	attempted	succeeded	MG	K 4a	other
0 14	22	12	1	3	9	19	15	0	4	12
15 86	8	4	1	0	4	13	11	4	2	9
Total	30	16	2	3	13	32	26	4	6	21

agglutinins and *M pneum* antibodies This negative correlation is not significant Three out of the 62 patients in this study developed a significant rise in titre of *Strep MG* agglutinins n h strep were not isolated from two of these in the third patient a strain of *Strep K 4a* was identified The number of *Strep K 4a* isolated in the serologically negative groups was not significantly different from that in the serologically positive groups

TABLE 2

Isolation of Streptococcus MG *and* Streptococcus K 4a *from 62 Patients in Relation to* Streptococcus MG *Agglutinins and Antibodies against* Mycoplasma Pneumoniae

Antibodies	Titre	Number of patients	Patients with isolation of Strep MG	Strep K 4a
Strep MG (aggl)	< 16	42	6	6
	≧ 16	20	0	3
M pneum (FAT)	<160	42	6	6
	≧160	20	0	3

FAT = *Indirect fluorescent antibody technique*

Simultaneous attempts were made to isolate both n h strep and *M pneum* from the 62 patients (Table 3) In the group of 11 patients from whom *M pneum* was isolated 9 strains of n h strep were isolated including three strains of *Strep MG* and three strains of *Strep K 4a* In the group of 51 patients from whom *M pneum* was not isolated three strains of *Strep MG* and six strains of *Strep K 4a* among a total of 40 strains of n h strep were isolated The number of *Strep MG* and *Strep K 4a* strains isolated in the *M pneum* positive group is not significantly different from the number of these strains isolated in the *M pneum* negative group

TABLE 3

Isolation of Non Haemolytic Streptococci and Mycoplasma pneumoniae *from 62 Patients*

	Patients	Number of strains of non haemolytic strep MG	K 4a	other	No isolation of non h strep
Isolation of M pneum	11	3	3	3	3
No isolation of M pneum	51	3	6	31	17
Total	62	6	9	34	20

The two age groups included in the material (41 patients 0–14 years old and 21 patients 15–86 years old Table 1) did not show any significant difference with regard to the number of n h strep isolated *Strep*

MG was isolated more frequently from adults than from children (P~ 0 03)

The intervals from onset of illness until attempts were made to isolate n h strep were on an average the same in the group with isolations as in the group without isolations 33 of the 49 strains were isolated during the second and third weeks after onset of illness

DISCUSSION

N h strep were isolated from more than half of the 62 patients *Strep MG* and *Strep K 4a* together representing about 1/3 of all the strains The proportion of isolations in the group of patients with p a p or virus pneumonia and in the group with other or absence of respiratory infections was not found to differ significantly However the limited number of patients and the mode of selection (13) do not permit conclusions to be drawn as to the role of *Strep MG* or *Strep K 4a* in p a p In the *M pneum* isolation study (13) six of the patients from whom *M pneum* was isolated developed a significant rise in titre of *M pneum* antibodies giving evidence that *M pneum* may have been of aetiological significance in these patients Isolation of n h strep from four of these patients was attempted resulting in the isolation of *Strep K 4a* from two of one non classified strain from the third no n h strep was isolated from the fourth of the patients Rise in titre of *Strep MG* agglutinins occurred in two of the four patients *viz* in one from whom *Strep K 4a* was isolated and in one from whom a non classified n h strep was isolated This lack of correlation is in agreement with the results obtained in 1944 in extensive bacteriological studies of patients with p a p published by *Dingle et al* (9)

In a study of volunteers inoculated with *M pneum* by *Rifkind et al* (21) *Strep MG* was isolated from just over 40 per cent of volunteers with or without *M pneum* antibody prior to inoculation and in the same frequency from those with and without illness Analyses of this study and of a subsequent *M pneum* inoculation study (7) showed that a rise in titre of *Strep MG* agglutinins occurred in 5 out of the 21 volunteers from whom *M pneum* was isolated and in 4 out of the 36 from whom *M pneum* was not isolated Similar results had been obtained earlier (5)

It has been shown (20) that *Strep K 4a* is immunologically closely related to *Strep MG* These two n h strep were encountered with almost the same frequency in the group of patients with p a p or virus pneumonia as in the group with other or absence of respiratory infections *Strep MG* was isolated more frequently from adults than from children

The number of *Strep K 4a* strains isolated represents a minimum as the semiselective medium employed contained sulphapyridine After the criteria for the selection of n h strep to be used in this study were

chosen it was found that 2 out of 10 *Strep K 4a* strains were sensitive to sulphonamide (20). However the bacteriostatic action of sulphapyridine in the semiselective medium was probably to some extent reduced as both specific (para aminobenzoic acid) and non specific (peptones) potential antagonists were also present in the medium.

No correlation was found between patients presenting a positive *Strep MG* agglutinin test and patients from whom *Strep MG* or *Strep K 4a* were isolated (Table 2). The amounts of sera were not sufficient for testing for *Strep K 4a* agglutinins.

On the other hand in the *M pneum* isolation study (13) a significant correlation between positive tests for *Strep MG* agglutinins and positive tests for *M pneum* antibodies was found: out of 21 patients with a positive test for *M pneum* antibodies 19 had a positive *Strep MG* agglutinin test and out of 56 patients with a negative test for *M pneum* antibodies 47 had a negative *Strep MG* agglutinin test.

The significance of the development of *Strep MG* agglutinins in patients with p a p has often been the object of discussion (23, 11, 8, 16, 6, 21, 17). Whether *Strep MG* should play any concomitant aetiological role in infections caused by *M pneum* has not been proved and seems doubtful. The development of *Strep MG* agglutinins in a patient with pneumonia due to *M pneum* is supposed to be due rather to antigenic relationship(s) between *Strep MG* and *M pneum*. The possibility that *M pneum* should be a stable L-form of *Strep MG* has been considered and tentatively rejected by serological comparison (17). Furthermore analyses of genetic relationship (18) gave evidence that *M pneum* could not be related to the L-form of *Strep MG*.

Experiments have shown (14) that absorption of p a p convalescent sera with *M pneum* significantly reduces *Strep MG* agglutinins and antibodies reacting with *Strep MG* by the FAT. By the absorption the homologous antibodies against *M pneum* were eliminated as estimated by the FAT and by the indirect haemagglutination test. However in most of the experiments the absorption with *Strep MG* gave a slight insignificant reduction of the antibodies against *M pneum* while the *Strep MG* antibodies were clearly reduced. These results indicate that antigenic components of *Strep MG* responsible for its reaction with p a p convalescent sera are related to some of the antigenic components of *M pneum*. It is probably the latter antigenic components which in patients with *M pneum* infections may elicit agglutinins against *Strep MG*.

CONCLUSION AND SUMMARY

Throat swabs from patients with primary atypical pneumonia have been examined for non haemolytic streptococci including *Streptococcus MG* and the immunologically closely related *Streptococcus K 4a*. The frequency with which these bacteria were isolated in the group of

patients with primary atypical pneumonia is not significantly different from that found in the group of patients with other or absence of respiratory infections The development of agglutinins against *Strepto coccus MG* in patients with pneumonia caused by *M pneumoniae* is possibly referred to related antigenic components of the two micro organisms

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IMMUNOLOGICAL RELATIONSHIPS BETWEEN *MYCOPLASMA PNEUMONIAE* AND *STREPTOCOCCUS MG*

By

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Received 5 ix 67

During studies on the aetiology of primary atypical pneumonia (p a p) in 1944 *Eaton et al* (3) recovered an agent which was later identified as a mycoplasma (1) and called *Mycoplasma pneumoniae (M pneum)* (2) At the same time a non haemolytic streptococcus designated *Streptococcus MG* (*Strep MG*) was studied in relation to the aetiology of p a p *Strep MG* was isolated in 1943 by *Thomas et al* (20) from the lungs of a patient who died from p a p Development of agglutinins against this streptococcus was demonstrated in a proportion of pa tients with p a p (21 4) In 1955 *Liu et al* (11) adapted the indirect fluorescent antibody technique to permit the demonstration of the Eaton agent *(M pneum)* in infected chick embryos and the titration of specific antibodies in sera from patients with p a p Absorption of such sera with *Strep MG* reduced the titre of antibodies against the Eaton agent This reduction however was later shown to be due to absorp tion of a factor present in fresh convalescent sera that enhanced the reaction of the specific *M pneum* antibodies which were not absorbed as demonstrated by the indirect fluorescent antibody technique this factor had many properties similar to complement and could be de monstrated in fresh normal human and guinea pig serum (*Liu* 1961 (14)) *Liu et al* (13) also showed that rabbits immunized against *Strep MG* had no antibodies reactive with the Eaton agent conversely rabbits immunized with the Eaton agent failed to develop *Strep MG* agglutinins They concluded that the *Strep MG* agglutinins and the Eaton agent antibodies are not related

Marmion & Hers (15) observed a slight reaction by complement fixation and immunofluorescence tests between *M pneum* and rabbit antiserum against *Strep MG*

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In attempts to elucidate possible immunological relationships between *M pneum* and *Strep MG*, cross absorption experiments were carried out a preliminary report of these has been published (8)

MATERIAL AND METHODS

Sera

Sera from six patients with p a p were stored at —20 C for up to 7 years They showed high titres of antibodies against *M pneum* and *Strep MG* when tested by the methods described below The sera were used either fresh thawed or heat inactivated at 56 C for 30 min In some of the absorption experiments normal human serum was added either fresh (FNS) or inactivated (56 C/30 min) (INS) The term normal refers to its negative reaction by all the immunological tests described below Rabbit antisera against *M pneum* were prepared as described elsewhere (10) Antisera against *Strep MG* were produced in rabbits by repeated intravenous injections of a heat killed and washed culture of the reference strain (No 8037 NCTC) grown for 18 hours in Todd Hewitt broth (18)

Antigens

When used for absorption the Mac strain of *M pneum* was prepared as described for the indirect haemagglutination (IHA) test (10) with omission of the sonication When used for precipitation tests the antigens were either sonicated *M pneum* (10) or HCl extracts of *M pneum* In the latter case the antigen was prepared as for absorption mixed with 0 1 N HCl and placed in a boiling water bath for 10 min after cooling neutralization with NaOH and centrifugation at 40 000 g for 15 min the supernatant was ready for use

The reference strain of *Strep MG* was grown for 18 hours in Todd Hewitt broth heat killed at 65 C for 60 min and washed three times in physiological saline This antigen was used as agglutinogen (21) and as absorbent

Streptococcus K 4a Streptococcus salivarius type I (strain 8606) and *Streptococcus salivarius* type II (strain Packham) were provided by Dr *B Perch*[1] (18) Antigens of these were prepared as for *Strep MG*

HCl extracts of *Strep MG* according to the method described by *Lancefield* (5) modified by the use of 0 1 N HCl were made by Dr *Perch* other extracts were made by freezing and thawing the organisms in saline suspension five or ten times

Diluents

When the effect of fresh normal serum (FNS) was investigated veronal buffered saline with Ca and Mg pH 8 was used as a diluent to give optimal conditions for the the action of complement (C) otherwise physiological saline was used

Absorptions

Sera were used either inactivated or untreated In some of the experiments they were diluted 1 15 or 1 ? prior to absorption After centrifugation of the antigen suspensions aliquots of the serum corresponding to 5 to 10 times the volume of the antigen sediment were added together with a few glass beads The mixture was shaken mechanically for 1 hour at room temperature and then centrifuged at 40 000 g for 30 min For reabsorption the supernatants were transferred to respective fresh antigen sediments shaken as before and left overnight at 4 C An aliquot of serum which was not absorbed but otherwise treated in the same way served as a control After centrifugation at 40 000 g for 30 min the supernatants were tested by the following techniques

Immunological Tests

The technique of the *indirect fluorescent antibody test* (FAT) has been described previously (7) It was applied to the titration of antibodies against *M pneum* and

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Strep MG. In the latter case antigens were prepared by placing a drop of *Strep MG* agglutinogen on slides. After air drying submersion in acetone for 10 min and re-hydration they were ready for use.

Titration of antibody against *M pneum* was also performed by an *indirect heamagglutination* (IHA) test (10).

Precipitation tests were carried out by double diffusion in agar gel by the method described by *Ouchterlony* (17). The wells were filled two or three times.

RESULTS

In all experiments with human sera absorption with *Strep MG* or *M pneum* greatly reduced the titre of antibodies against the homologous organism as measured by the serological techniques described. Another constant result was a significant reduction of antibodies against *Strep MG* on absorption with *M pneum*. Absorptions with *Strep MG* gave a slight or no reduction in the anti *M pneum* titres. In cases where a reduction of anti *M pneum* titre by the absorption with *Strep MG* was found this reduction was independent of the presence during the absorption of fresh normal (FNS) or inactivated normal (INS) human serum. The reduction of anti *M pneum* titre was also independent of the use of FNS or INS as diluent for the absorbed serum in the FAT. This independence was demonstrated even when an antiglobulin conjugate was used which in the presence of FNS increased the titre of *M pneum* antibodies. Furthermore the reduction of *M pneum* antibodies after absorption with *Strep MG* was demonstrated by the IHA test albeit less clearly and less often. In two experiments where this absorption resulted in at least a 4 fold reduction of *M pneum* antibody titre demonstrated by the FAT absorption with *Streptococcus salivarius* type II which is immunologically non related to *Strep MG* (18) did not affect the anti *M pneum* titre (Table 1).

When human p a p sera were inactivated prior to absorption *Strep MG* agglutinins could either not be detected or were greatly reduced; this has been pointed out by *Mirick et al* in 1944 (16). After inactivation of the sera *Strep MG* agglutinin titres could not be restored to their former levels by the addition of FNS or INS. Inactivation did not affect antibodies against *Strep MG* reacting in the FAT.

One serum was also tested for agglutinins against two non haemolytic streptococci which are immunologically related to *Strep MG* viz *Streptococcus K 4a* and *Streptococcus salivarius* type I (18). The serum agglutinated *Strep MG* to a titre of 90 while the agglutinin titres with *Streptococcus K 4a* and *Streptococcus salivarius* type I were 45 and 60 respectively. After absorption with both *Strep MG* and *M pneum* the titres of agglutinins against all three streptococcal strains were reduced to below 16 (Table 1).

After absorption of human sera with *M pneum* the IHA titre of *M pneum* antibodies was generally reduced by a factor 10 –10. The impression that this reduction was not due to an inhibition of the IHA reaction by soluble antigen remaining in the serum after absorption

TABLE 1

Absorptions of Two Sera from Primary Atypical Pneumonia Patients with Mycoplasma pneumoniae *and* Streptococcus MG

Serum A and B absorbed with		Titre of antibodies against					
		Mycoplasma pneumoniae		Streptococcus MG		Strep K 4a	Strep saliv type I
		FAT	IHA	FAT	Aggl	Aggl	Aggl
Nil	A	2800	15 000	360	90	45	60
	B	1280	2560	160	24	NT	NT
M pneum	A	<16	<16	<35	<16	<16	<16
	B	≦10	<10	≦10	< 8	NT	NT
Strep MG	A	360	2240	<32	<16	<16	<16
	B	320	2560	<40	< 8	NT	NT
Strep saliv type II§	A	1600	15 000	290	72	36	72
	B	1280	2560	120	16	NT	NT

FAT = Indirect fluorescent antibody test
IHA = Indirect haemagglutination test
Aggl = Tube agglutination test
Immunogically related to *Strep MG*
§ Immunologically unrelated to *Strep MG*
NT = Not tested

was substantiated by testing a mixture of equal parts of absorbed and non absorbed serum the titre of the latter was reduced only parallel with the dilution by the former By means of HCl extracts and freeze thaw extracts of *Strep MG* it was attempted to inhibit the *M pneum* antibodies reacting in the IHA test From various dilutions of these extracts one drop was added to each cup of parallel dilutions of a positive serum the mixtures were allowed to react for 30 min at room temperature The cells sensitized with *M pneum* antigen were then added as described for the IHA test (10) A sonicated *M pneum* antigen was tested for inhibitory potency in the same way While the *M pneum* antigen significantly inhibited the reaction no inhibition was obtained with the *Strep MG* extracts

Sera from rabbits immunized against *M pneum* did not react with *Strep MG* neither by agglutination nor in the FAT Anti *Strep MG* sera from hyperimmune rabbits did not react with *M pneum* antigen in the FAT or IHA test This is in accordance with the data given by *Liu et al* in 1959 (13)

Precipitations in agar gel were formed by all the extracts of *Strep MG* and the homologous antiserum The sonicated *M pneum* antigens reacted with anti *M pneum* rabbit sera by forming from one to four precipitation lines while HCl extracts developed only one line No precipitation line was formed by extracts of *Strep MG* and rabbit anti *M pneum* serum nor did the sonicated or HCl extracted *M pneum* anti

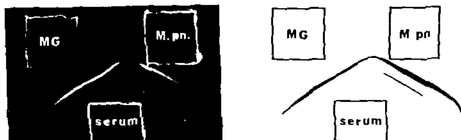


Fig 1
Freeze thaw extra t of *Strep MG* and soni ated *M pneum* precipitating human p a p serum with reaction of identity

gens precipitate the anti *Strep MG* sera from hyperimmunized rabbits Human sera with high antibody titres in the other serological tests described reacted with the *Strep MG* extracts forming one two or three precipitation lines One or two faint precipitation lines occurred both with the sonicated and with the HCl extracted *M pneum* antigens and the human sera but only if present in optimal antigen/antibody concentration A reaction of identity (17) was demonstrated between the extracts of *M pneum* and *Strep MG* by their lines developed with two human p a p sera (Fig 1)

DISCUSSION

The results of the absorption and precipitation experiments indicate that antigenic components of *Strep MG* responsible for its reaction with p a p sera are closely related to some of the antigenic components of *M pneum*

Mirick et al (16) showed that the capsule of *Strep MG* contains a polysaccharide antigen which is responsible for its type specific serological reactions *e g* by agglutination and precipitation HCl extracts of streptococci contain mainly polysaccharides (5) Such extracts of *Strep MG* readily precipitated homologous rabbit antisera and less readily some of the human p a p sera

Sobeslavsky et al (19) investigated a number of chemical and chromatographic fractions of *M pneum* for their antigenicity and serological reactivity Their findings suggested that the sensitizing moiety in the IHA is a lipoprotein the protein component is mainly responsible for adsorption to tanned red cells whereas the lipid component is mainly responsible for the reaction with specific agglutinating antibodies The lipid containing fractions which were also active in complement fixation appeared to block the growth inhibition effect of antiserum as measured by the tetrazolium reduction inhibition test These activities were not found in the polysaccharide fractions which were chiefly responsible for the precipitation activity

The immunological relationships of *M pneum* and *Strep MG* de

monstrated in the present study are possibly due in part to closely related carbohydrate antigens. This may be the case with the HCl extracts in the immunoprecipitation tests. The ability of *M. pneum.* to absorb human serum agglutinins against three immunologically related streptococci, viz. *Strep. MG*, *Streptococcus K. ra* and *Streptococcus salivarius* type I, is possibly also due to related carbohydrate components of the micro-organisms.

The suggestion by *Sobeslavsky et al.* that a lipid component of *M. pneum.* is responsible for the specific antibody reaction in the IHA test cannot be directly referred to the IHA test of the present study which differs from the former in that formalinized sheep red cells were used. However, the inability of HCl or saline extracts of *Strep. MG* to inhibit the reaction of anti *M. pneum.* in the IHA test may fit in with their suggestion.

The nature of *M. pneum.* antigen reacting in the FAT has not, to the author's knowledge, been investigated.

Most probably the organisms contain more than one related antigenic component. Perhaps one of these is not exposed as a site on the whole cell of *Strep. MG*. This might explain why the reduction of *M. pneum.* antibodies on absorption with *Strep. MG* was only slight and in some experiments even lacking. The reduction was independent of the presence of FNS during the absorption procedure; it was also independent of the presence of FNS in the diluent when the absorbed sera were titrated for the FAT. This is not in agreement with the absorption experiments carried out by *Liu* (12, 14) who found this reduction to be due to absorption of a complement-like factor present in fresh p.a.p. sera; this factor enhanced the reaction of the *M. pneum.* antibodies which were not absorbed, as demonstrated by the FAT. The disagreement with the present results may be due to differences in the antigenic composition of the *Strep. MG* prepared for absorption, or to differences in the sera absorbed.

As yet the aetiological role of *Strep. MG* in p.a.p. is unclear; most authors seem to consider the development of *Strep. MG* agglutinins in this condition to be due to antigenic relationships between *Strep. MG* and *M. pneum.*, the latter having been proved to be the aetiological agent in a proportion of primary atypical pneumonias (for references see 9). This point of view is supported by the experiments reported here. The relationship between these otherwise non-related micro-organisms may be parallel to, for example, that of *Proteus vulgaris* types OX 19 and OX 2 and some of the Rickettsiae. Rabbits vaccinated with killed *M. pneum.* did not elicit antibodies against *Strep. MG*. Perhaps such antibody production would depend on the presence of living or multiplying *M. pneum.* organisms, which may be the case in *M. pneum.* infections in man. To the author's knowledge, development of *Strep. MG* agglutinins has not been described in connection with vaccination of human subjects with a killed strain of *M. pneum.*

SUMMARY

Immunological relationships of *Mycoplasma pneumoniae* and *Streptococcus MG* were demonstrated by cross absorption experiments on sera from patients with primary atypical pneumonia It is suggested that the *Streptococcus MG* agglutinins present in some of the sera from patients with infection due to *Mycoplasma pneumoniae* might have been elicited by antigenic components shared by these microorganisms

ADDENDUM

Since this work was completed related serological investigations have been described in Aust J exp Biol med Sci 45 163–187 1967 by *B P Marmion* *I Plackett* & *Ruth Lemcke* in a paper entitled Immunochemical analysis of *Mycoplasma pneumoniae* I Methods of extraction and reaction of fractions from *M pneumoniae* and from *M mycoides* with homologous antisera and with antisera against *Streptococcus MG* They demonstrated reactions between *M pneum* antigens and rabbit antiserum against *Strep MG* both by precipitation in gel and by complement fixation while they were unable to show the reciprocal reaction between *Strep MG* antigen and antisera to *M pneum* After absorption of a rabbit anti *Strep MG* serum with a chloroform methanol extract or whole cells of *M pneum* they found the *Strep MG* agglutinin unaltered

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ANTIBODY FORMATION IN CELL CULTURES

1 *Membrane Cultures of Lymphoid Cells a System for Studies of Long Term Secondary Antibody Responses to Poliovirus*

By

SVEN ERIC SVEHAG[1] BRUCE CHESEBRO and PER KVARNFORS[3]

Received 3 xi 67

The embedding of tissue fragments from lymphopoietic organs of immune animals in agar or plasma clots bathed in suitable media has in recent years permitted qualitative study of certain aspects of antibody formation *in vitro* Furthermore the initiation of secondary (*Stavitsky* 1960 *Michaelides & Coons* 1963 *Ambrose* 1963 *Uhr* 1963 *Tao* 1964 *Medzon & Vas* 1964 *Halliday & Garvey* 1964 *Svehag* 1965 *Juhasz & Rose* 1965 *Kritzman & Harper* 1966 *Ortiz Muniz & Sigel* 1967) as well as primary (*Fishman* 1959 *Saunders & King* 1966 *Tao & Uhr* 1966 *Globerson & Auerbach* 1966) *in vitro* antibody responses in such fragments by restimulation with different antigens has been reported

Although the use of organ cultures has permitted studies of the overall production of antibodies by tissue fragments and identification of antibody producing cells by the use of immunofluorescent techniques fragment cultures have certain limitations for quantitative studies On the other hand the use of isolated cells provides a more uniform environment for the cultured cells and eliminates retention of interstitially stored antibody Consequently many attempts have been made to cultivate suspended lymphoid cells capable of antibody formation (*Michaelides & Coons* 1963 *Svehag* 1965 *Wesslen* 1952 *Steiner & Anker* 1956 *Vaughan et al* 1960 *Hallander & Danielsson* 1962) and recent improvements of the cultivation technique have rendered it possible to follow the antibody synthetic process in such cultures for several days (*Dutton* 1967 *Mishell & Dutton* 1966 *Nilsson* 1967)

Steiner & Anker (1956) utilized a cultivation technique earlier employed in organ culture studies namely the deposition of the cells on

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a membrane in a gas—liquid interphase With this procedure secondary antibody formation could be maintained for a few days by isolated spleen cells We will here report on long term secondary antibody responses *in vitro* to poliovirus (PV) using a membrane cultivation technique earlier described by us (*Svehag* 1965) In particular we have studied the quantitative and qualitative aspects of antibody formation induced by greatly differing antigen multiplicities the effect of heterologous antigen and phytohaemagglutinin on the antibody response and the morphological changes occurring in cell cultures during antibody formation

MATERIALS AND METHODS

Antigens The Brunhilde strain type 1 of PV was used The virus was grown on HeLa cell monolayers and virus stocks were partly purified by differential centrifugation and banding in CsCl solutions (average density 1 34) prior to storage at —20 C. The infectivity of virus stocks was assayed on HeLa monolayers and expressed in plaque forming units (PFU) per ml virus suspension The Edmonton strain of measles virus[1] was propagated in a cell line (Lu 106) of human lung embryo origin Haemagglutination tests were performed according to the method described by *Rosen* (1961)

Immunization procedure Four months old rabbits were immunized intravenously with 10^8–10^9 PFU poliovirus at various times prior to sacrifice

Spleen cell cultures Four to fourteen days after an immunization with poliovirus rabbits were splenectomized aseptically under nembutal anaesthesia and spleens were placed in ice cold medium Eagle in Earle s buffer or Parker 199 supplemented with 15 per cent normal calf serum and antibiotics The organs were cut into small fragments washed in medium and dispersed in a Borell apparatus The separated cells were filtered through gauze into a centrifuge bottle containing 6 per cent Dextran 150 and 0 45 per cent EDTA in physiological saline buffered with 0 01 M phosphate to pH 7 3 (PBS) The cell suspension was centrifuged in the cold for 10 minutes at about 3000 C The top fraction of the packed cells containing granulocytes and lymphoid cells was collected the cells counted and 1 to 2 × 10^7 cells were seeded on Millipore membrane filters (0 45 mμ) which had previously been glued to the top of lucite cylinders Each cylinder was placed in a depression of a plastic tray 0 5 ml of medium was added and the cells were covered with a piece of porous paper which reached down into the medium The tray was finally covered with a plastic film and placed in a 37 C incubator which contained humidified 5 per cent CO in air The culture medium was replaced every second or third day and titrated for PV neutralizing antibody by the plaque inhibition technique on HeLa monolayers The specificity of the *in vitro* response was established by assaying the media against serologically unrelated viral antigens

Staining of cells from membrane cultures A sample of cultured cells was withdrawn from the membrane with the aid of a Pasteur pipette suspended in Parker 199 and sedimented onto a glass slide in a specifically designed centrifuge rotor The slide was fixed and stained with methyl green pyronin (T G Gurr London England) or May Grunwald Giemsa

Antibody titrations Sera and cell culture media were titrated for poliovirus neutralizing antibody by mixing equal volume of virus (100 PFU) and varying dilutions of serum or medium. The mixtures were held for 4 hours at room temperature plus overnight at 3 C and were assayed without dilution on HeLa monolayers for virus survivors The antibody activity was expressed in per cent virus neutralization or in 50 per cent neutralizing units defined as the serum dilution at which the plaque formation was reduced by half

When measuring antibodies to measles virus the haemagglutination inhibition tests were carried out according to the method of *Rosen* (1961)

Zonal density gradient centrifugation Linear gradients of sucrose dissolved in

[1] kindly supplied by Dr Erling Norrby

PBS in concentrations ranging from 10 to 37 per cent were prepared by the use of a mixing device 0 2 ml of serum or concentrated medium was layered on a 4 7 ml gradient and the gradient was centrifuged in a Spinco model L centrifuge employing a SW 39 rotor at a maximum centrifugal force of 135 000 G for 22 hours Fractions (0 2 to 0 3 ml) were collected dropwise through a hole in the bottom of the tube Protein determinations and antibody titrations were performed on each fraction

Reduction and alkylation of antibodies Tissue culture media were incubated with equal volumes of PBS containing 0 2 M 2 mercaptoethanol at room temperature for 3 hours and subsequently alkylated for 40 minutes at 3 C with 0 04 M iodoacetic acid in 0 01 M Tris HCl buffer pH 8 0 Control samples were incubated with only PBS or iodoacetic acid To remove excess reagents all samples were dialysed in the cold against PBS

Immunoelectrophoresis The microtechnique of *Scheidegger* (1955) was used

Gel diffusion analysis These tests were performed according to the microtechnique described by *Wadsworth* (1957)

Protein determination Protein concentrations were measured by absorbance at 280 mμ in a Beckman DU spectrophotometer in a 1 0 cm wide quartz cuvette

Ultraviolet light inactivation of virus Poliovirus was inactivated by ultraviolet light (UV) in a constantly agitated Petri dish placed 20 cm from a 15 watt Sylvania germicidal lamp This treatment resulted in a reduction of six $\log_{10}$ of virus infectivity in 4 minutes

In vitro stimulation with antigen Fractionated spleen cell suspensions were mixed with various antigen multiplicities (physical virus particles/cell) of CsCl banded and UV irradiated PV The cells were incubated for 30 minutes at 37 C, washed twice counted and seeded on membrane filters Controls included spleen cells incubated with measles virus or PBS

RESULTS

Biphasic Antibody Responses by Spleen Cells Fractionated in Dextran

A rabbit which had received 4 × 10^8 PFU of virus intravenously 14 days earlier was splenectomized and the cell suspension centrifuged in Dextran 150 The packed cells were divided into 5 fractions numbered I II III IV and V from the top and the capacity of the 4 top fractions to respond with antibody formation upon antigenic restimulation *in vitro* was investigated The number of cells per membrane culture was 10^7 and the antigen multiplicity was 25

Cell fraction II gave the most rapid antibody response—in these cultures an increase in virus neutralizing activity was detectable 5–6 days after stimulation with antigen (Fig 1) The antibody response of fractions I II and III was biphasic while fraction IV did not respond with detectable antibody formation

The first antibody peak was obtained 10–12 days after stimulation the second 3 weeks after incubation with antigen The first antibody peak contained primarily 2 mercaptoethanol sensitive antibody which was gradually replaced by antibody resistant to reduction during the formation of the second antibody peak The top cell fraction (I) was responsible for the longest antibody response which lasted more than 6 weeks (Fig 1) Treatment with 2 mercaptoethanol indicated that these antibodies were primarily IgG This was corroborated by immunoelectrophoresis In addition a strong line in the α globulin region was obtained with media from all stimulated as well as unstimulated cultures The significance of this α globulin synthesis is unknown

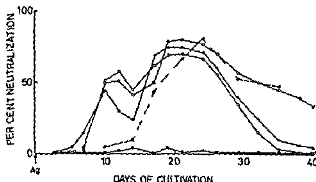


Fig 1

Secondary in vitro antibody responses by Dextran fractionated rabbit spleen cells The animal had received 4 × 10⁸ PFU of poliovirus intravenously 14 days prior to splenectomy The cells were restimulated with an antigen multiplicity of ⁰5 prior to cultivation and the media were assayed for antibodies at a dilution of 1 3 O-cell fraction I △ fraction II ▢ fraction III fraction IV ● fraction I after reduction of the antibodies by 2 mercaptoethanol

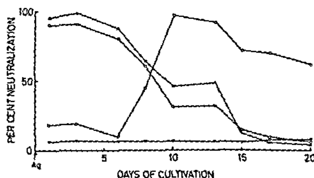


Fig 2

Interference of transferred antibody with the induction of in vitro antibody responses The animal had received two injections of 3 × 10⁸ PFU of poliovirus intravenously 21 and 4 days prior to splenectomy The cells were restimulated with an antigen multiplicity of 15 one sample was extensively washed with PBS prior to stimulation The media were tested at a dilution of 1 3 ▢ and △ cells not washed prior to cultivation O washed with PBS medium only

In occasional experiments control cell cultures from immunized animals showed an unexpected slight increase of virus neutralizing activity 7 to 10 days after cultivation even in the absence of antigenic restimulation Such spontaneous transient 19S responses were also observed during the second week in culture in subsequent experiments

Interference of Transferred Antibody with the Induction of in vitro Antibody Responses

Splenectomy was performed on a rabbit which had received two injections of 3 × 10⁸ PFU of PV 3 weeks and 4 days prior to the splenectomy The serum antibody titre primarily associated with 7S glob

ulins was 10^4 50 per cent neutralizing units The cells were fractionated in Dextran and the top fraction was divided into three aliquots all of which were stimulated with PV (antigen multiplicity = 15) one fraction after extensive washing with PBS The cells were cultivated employing 2×10^7 cells/membrane

Only lymphoid cells which had been carefully washed prior to antigenic stimulation gave rise to normal *in vitro* antibody responses at the expected time that is 7 to 10 days after stimulation (Fig 2) The responses of the remaining cultures were very weak and transitory These latter cultures in contrast demonstrated a considerable release of transferred antibody into the culture medium during the first 6 days of cultivation The inhibition of the secondary antibody response using cell fractions not washed to remove transferred antibody prior to restimulation with homologous antigen was particularly marked when the serum 7S antibody titre was high

Potentiation of Secondary in vitro *Antibody Responses by Phytohaemagglutinin*[1]

The effect of incorporating phytohaemagglutinin (PHA) into the culture medium on *in vitro* antibody responses was investigated in the following type of experiments

A rabbit which had received 3×10^8 PFU of PV intravenously 21 and 4 days before was splenectomized the spleen cells were fractionated in Dextran and the top fraction divided into three aliquots Two aliquots were incubated with a low antigen multiplicity (about 2) while the third aliquot was not stimulated The cells were cultivated in Parker 199 and 15 per cent calf serum one of the stimulated aliquots and the unstimulated one received 0 5 ml of rehydrated PHA/100 ml medium

The incorporation of PHA into the medium resulted in a significantly shortened induction period higher antibody titres and a prolonged antibody response (Fig 3) Treatment with 2 mercaptoethanol indicated that the response contained both 19S and 7S antibodies All cell cultures antigen stimulated as well as not stimulated showed a pronounced blastoid cell transformation in the presence of PHA

Effect of Antigen Multiplicity on Molecular Type of Antibody Synthesized and Duration of the Antibody Response

Two factors were mainly responsible for the molecular type of antibody contained in the *in vitro* antibody responses and the duration of these responses namely the immunization schedule used for *in vivo* sensitization and the antigen multiplicity employed for *in vitro* stimulation The importance of the latter factor is illustrated by the following experiment

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[1] Phytohaemagglutinin M D fco

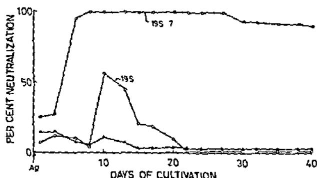


Fig 3

Potentiation of a *secondary in vitro* antibody response by phytohaemagglutinin The rabbit had received 3 × 10^8 PFU of poliovirus intravenously 21 and 4 days prior to splenectomy The Dextran fractionated cells were divided into three aliquots □ restimulated with an antigen multiplicity of 2 0 5 ml rehydrated PHA/100 ml medium △ not restimulated 0 5 ml rehydrated PHA/100 ml medium ○ restimulated with an antigen multiplicity of 2 no PHA The media were tested at a dilution of 1 3

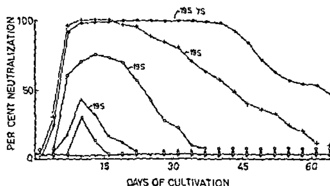


Fig 4

Effect of antigen multiplicity on duration of the *in vitro* antibody *response* A rabbit had received 3 × 10^8 PFU of poliovirus intravenously 6 days *prior* to splenectomy The cells were divided into five aliquots and incubated with poliovirus multiplicities of + 20 ○ 2 △ 0 2 □ 0 02 and 0 002 A second rabbit had received 3 × 10 PFU 2 weeks prior to surgery The antigen multiplicity used in vitro was 40 (●) The media were tested at a dilution of 1 3

A rabbit was given 3 × 10^8 PFU of PV intravenously 6 days *prior to* splenectomy The animal was bled prior to the surgery and the serum antibody was found to be predominantly of the 19S *type* The spleen cells were centrifuged in Dextran a top fraction (¼ of the packed cells) was recovered and divided into five aliquots Each aliquot containing 6 × 10^7 cells was incubated with PV employing antigen multiplicities of 20 2 0 2 0 02 and 0 002 The cells were washed twice and seeded on Millipore *membranes* (2 × 10^7 cells/*membrane*) *Controls included* spleen *cells from* a nonimmunized rabbit and medium only The results of antibody titrations are given in Fig 4

Only stimulation with the four largest antigen multiplicities gave rise to detectable *in vitro* antibody response Stimulation with a multiplicity of 20 evoked a surprisingly rapid and rather strong 19S antibody response A gradual decrease of the antigen multiplicity caused a proportionate prolongation of the induction periods of the resulting responses and a reduction of their titre Only 19S antibodies were produced in response to the antigen multiplicities 20 2 and 0 2 The molecular type of antibodies formed after stimulation with the multiplicity 0 02 could not be determined due to the weakness of the response Its kinetics was however that of a typical 19S response

An even higher antigen multiplicity (40) was used in a similar experiment where the rabbit had been immunized with 3 × 10^8 PFU of virus 2 weeks prior to splenectomy The resulting *in vitro* antibody response containing both 19S and 7S antibodies was very high in titre and lasted for 2 months (Fig 4)

Inability of Heterologous Antigen Stimulation to Interfere with the in vitro *Antibody Response to Homologous Antigen*

It was investigated whether the incubation of sensitized lymphoid cells with heterologous antigen would influence their capacity to respond to subsequent stimulation with homologous antigen

Splenectomy was performed on a rabbit which had received intravenous injection of PV (7 × 10^8 PFU) three months earlier The rabbit was bled before the splenectomy and the serum antibody titre was found to be completely unaffected by reduction with 2 mercaptoethanol The spleen cells were centrifuged in Dextran a top fraction from the packed cells was recovered and divided into four aliquots each containing 6 × 10^7 cells Each of the four aliquots was incubated with one of the following 1) 10^3 haemagglutinating units of measles virus for 30 minutes at 37 C and subsequently UV irradiated PV using an antigen multiplicity of 15 2) PBS plus PV 3) measles virus only and 4) PBS only The four cell suspensions were washed twice with PBS and seeded on Millipore membranes (2 × 10^7 cells/membrane) Controls included membranes with medium only The antibody activity of media from the various cell fractions was assayed by the plaque inhibition technique against PV and in the haemagglutination inhibition test against measles virus (Fig 5) It can be seen first that prior incubation of sensitized lymphoid cells with a large dose of heterologous antigen (measles virus) did not visibly alter their capacity to respond with *in vitro* antibody formation to the homologous antigen (PV) and second that incubation of the sensitized cells with heterologous measles virus antigen induced no detectable antibody response either against this antigen or against PV

The morphology of cells in membrane cultures during antibody formation Spleen cells were centrifuged in Dextran the packed cells

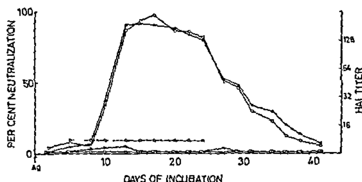


Fig 5

Inability of heterologous antigen stimulation to interfere with the in vitro antibody response to homologous antigen The animal had received 7×10^8 PFU of polio virus 3 months prior to splenectomy △ in vitro restimulated with measles virus and subsequently poliovirus O PBS and poliovirus □ measles virus only ▽ PBS Unbroken lines = poliovirus neutralizing antibody Broken line = antibodies to measles virus measured in hemagglutination inhibition tests The media were tested at a dilution of 1 4

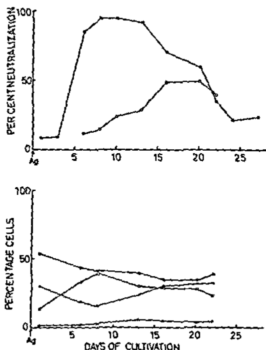


Fig 6

Relationship between antibody formation (top figure) and relative cell counts (bottom figure) The animal had received 5×10 PFU of poliovirus 21 days prior to splenectomy The cells were restimulated in vitro with an antigen multiplicity of 40 Three hundred cells were counted on each slide and the percentage of different cell types was estimated ● total antibody ○ 2 mercaptoethanol resistant antibody ■ lymphocytes small and medium □ blastoid cells △ plasma cells and ▲ mononuclear cells The medium was tested at a dilution of 1 4

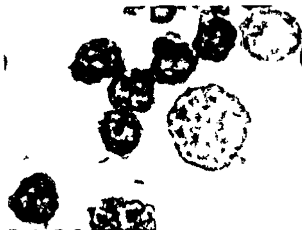


Fig 7
Smear from the top fraction of a Dextran gradient showing lymphocytes and a large mononuclear cell May Grunwald Giemsa × 5000 phase contrast microscopy

were divided into 5 fractions and smears prepared from each fraction were fixed stained and examined by phase contrast microscopy The distribution of cells in the fractions numbered I II III IV and V from the top were as follows fraction I mainly small and mediumsized lymphocytes and a few large mononuclear cells (Fig 7) fraction II many lymphocytes and some large mononuclear cells fraction III a few lymphocytes and erythrocytes and many large mononuclear cells fraction IV a few large mononuclear cells and plenty of erythrocytes fraction V mainly erythrocytes

In most of the *in vitro* studies only splenic cells from fraction I were cultivated since this fraction was rich in lymphocytes but occasionally both fractions I and II were utilized In the following experiment the morphology of the cells originating from these two fractions during antibody formation *in vitro* was studied Splenectomy was performed on a rabbit which had been given two intravenous injections of 5 × 10^{8} PFU of PV 3 weeks and 4 days prior to the operation The suspended spleen cells were centrifuged in Dextran and the two top fractions of the packed cells were recovered incubated with PV (antigen multiplicity = 40) and cultivated on Millipore membranes (10^{7} cells/culture)

At each change of medium cells were removed from selected cultures fixed and stained with methyl green pyronin Three hundred cells were counted on each slide and the percentage of the different cell types was estimated All media were assayed for PV neutralizing antibody some of them both prior to and after reduction with 2 mercaptoethanol The relationship between antibody formation and relative cell counts is shown in Fig 6 The cultures responded to antigenic stimulation with 19S antibody formation reaching a peak 8 to 10 days after the stimu

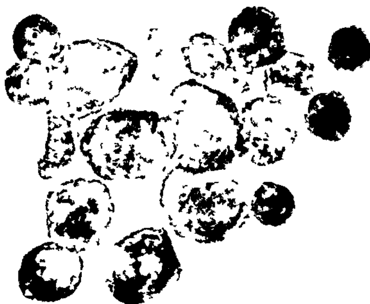


Fig 8

Smear 3rd day of cultivation Blastogenesis several normal lymphocytes are still seen Pyronine × 5000

lation followed by a weaker 7S antibody response with maximal titres on the 16th to 20th day Immunoelectrophoretic analysis of media taken on the 16th day revealed a clearly visible IgG line

10 to 15 per cent blastoid cells were observed in smears prepared as early as on the 2nd day of cultivation (Fig 6) These cells were 2 to 3 times the size of small lymphocytes (Fig 8) and their large nuclei contained one or a few nucleoli and a fine fibrillary chromatin pattern Mitotic figures were seen occasionally and the rather scanty cytoplasm was strongly pyroninophilic The blastoid cells were often seen to form clusters of various size Their relative number in the cultures increased to nearly 40 per cent on day 8 decreased to 25 to 30 per cent during the 2nd week and remained at this level for about two weeks The relative increase of blastoid cells appeared to coincide with a decrease in small lymphocytes and with the 19S antibody response Occasionally binucleated cells with rather large cytoplasm and reticulated nuclei were seen (Fig 9) A low percentage (2 per cent to 4 per cent) of plasma cells was observed from one week on

In certain experiments stained preparations from the cultures contained a different type of large pyroninophilic cells (Fig 10) These



Fig 9

Smear 3rd day of cultivation showing binucleated cell and two lymphocytes May Grunwald Giemsa × 8000

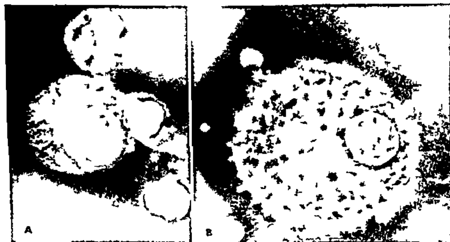


Fig 10

(a) Negative print of smear 2nd day of culti at h w ng a cell at an earl tag f tran formation May Crun al l Ciemsa 8000 phase contrast mi copy
(b) Negative print of smear t o week of cult t n th transforme l ells have increase l in size and their nuclei have an t e ppearance M Crunwald Giemsa × 8000 phase cont t microscopy



Fig 11
Smear one week of cultivation showing large transformed cells with small lymphocytes fixed to their surface May Grunwald Giemsa × 3000 phase contrast microscopy

cells had a large cytoplasma and a round or ovoid excentric nucleus with a clear or reticulated chromatin net After one or two weeks of cultivation these cells often became vacuolated They were not seen in the Dextran gradient prior to cultivation These large cells which often had dead or living small lymphocytes fixed on their surface (Fig 11) increased in number and in size from 3 to about 6 times the size of small lymphocytes with time of cultivation Two to four days after *in vitro* stimulation with antigen their relative number was 2 to 4 per cent as compared to 5 to 8 per cent after one week and about 20 per cent after two weeks When the cells were cultivated without antigenic stimulation but in the presence of PHA the percentage transformed cells increased more rapidly from 2 to 4 per cent on day two to 15 to 20 per cent after one week and 25 to 30 per cent after two weeks A few per cent of the large cells were observed even when the spleen cells were cultivated in the absence of antigenic restimulation or PHA The modal values for the size of the transformed cells at different times of cultivation as related to the size of small lymphocytes were after 2 to 4 days about three times small lymphocytes after one week 4 to 5 times small lymphocytes and after two weeks 5 to 6 times the size of the lymphocytes

It was previously mentioned that culture media sometimes showed an increase in virus neutralizing activity 7 to 10 days after the cells were placed in culture even when no *in vitro* stimulation with antigen was performed In one experiment where this occurred cell samples were prepared from the cultures fixed and stained with methyl green

pyronin Three hundred cells were counted per slide and the relative cell counts determined for the different cell types The antibody produced was sensitive to 2 mercaptoethanol treatment and the transient antibody response reached a peak on the 10th day of cultivation A rather high percentage (about 15) of blastoid cells were seen in the cultures as early as on 3rd day of cultivation This percentage remained constant up to day 8 but then increased to about 30 per cent This increase paralleled the 19S antibody response Occasionally cytoplasmic bridges were observed between mononuclear cells and small lymphocytes

DISCUSSION

The results show that it is possible to maintain isolated lymphoid cells of spleen origin in membrane culture for up to two months and that such cells when procured from a sensitized animal are capable of responding to renewed antigenic stimulation with specific antibody formation Until very recently it has not been possible to maintain the immunological activity of lymphoid cells in free suspensions for more than a few hours Recent improvements of techniques used for cultivation of lymphoid cells in suspension have emphasized the importance of cell density and the osmotic pressure of serum protein or macromolecular substances in maintaining the viability of the cells (For review see *Dutton* 1967)

In the present study the highly specific and sensitive plaque inhibition neutralization test was used The appearance of antibody following each change in medium was considered to represent *de novo* synthesis for the following reasons 1) carry over of antibody during replacement of medium was negligible 2) the antibody titre declined rapidly following freezing thawing of the cells or the addition of puromycin (0 5 μg/ml) actinomycin D (2 to 4 μg/ml) to the medium (*Svehag* 1964) 3) there is no indication that *in vivo* formed antigen antibody complexes could serve as a source of antibody subsequent to dissociation *in vitro* and 4) the cell cultures were metabolically active for several weeks as judged by acid production (*Svehag* 1965)

Durable *in vitro* antibody responses (up to 2 months) were observed when the response involved 7S antibody formation or when 19S formation was induced by high antigen multiplicities Immunization with carefully adjusted antigen doses has *in vivo* been reported to allow a partial separation in time of 19S and 7S antibody formation (*Svehag & Mandel* 1964) In the present study similar biphasic antibody responses were obtained *in vitro* following restimulation with rather high antigen multiplicities when the primary sensitization had induced the formation of both 19S and 7S antibodies The secondary *in vitro* 19S antibody responses reached a peak about 10 days after restimulation that is these responses were delayed when compared to the corresponding *in vivo* responses One of the factors responsible for this retardation

of the *in vitro* response is the great reduction (up to 60 per cent) of the number of viable cells which occurs during the first 48 hours after cultivation The starting level of competent cells is consequently very low when compared to in the whole intact animal

The *in vitro* 19S response to a small dose of antigen exhibits the same precipitous drop subsequent to attaining peak titres as the corresponding *in vivo* response (*Svehag & Mandel* 1964) In the *in vivo* system this effect has been interpreted in different ways It has been considered (1) to reflect a migration of 19S forming cells from the spleen to extrasplenic lymphoid organs (*Wissler & La Via* 1960) (2) to be the result of a feedback mechanism mediated by the simultaneous appearance of 7S antibodies (*Wigzell* 1967) or (3) to be due to a depletion of antigen (*Svehag & Mandel* 1964) As a alternatives (1) and (2) could be excluded in the present *in vitro* system when no 7S antibody formation was detectable a depletion of antigen or some other unknown mechanism remains as the explanation of the sudden interruption of the 19S antibody synthesis

The *in vitro* response also mimics the *in vivo* situation in one other aspect Stimulation of primed cells with antigen in the presence of specific antibodies particularly of the 7S type caused a partial inhibition of the *in vitro* response The likely explanation also arrived at in the *in vivo* studies (*Sahiar & Schwartz* 1964 *Rowley & Fitch* 1964) was that the viral antigen rapidly formed complexes with the transferred antibodies and that these complexes were less effective in inducing the cells to renewed antibody formation

It was noted that when the *in vivo* sensitization gave rise to only 19S antibody formation restimulation of the cells *in vitro* consistently evoked 19S but no 7S responses In addition it could be shown that incubation *in vitro* of primed cells with heterologous antigen did not adversely affect the capacity of these cells to respond with antibody formation to the homologous antigen These observations support the contention that the primed cells and their progeny upon restimulation produce predominantly antibody of the same molecular type and specificity as they did following the initial *in vivo* priming These data are consistent with the results of *Michaelidis & Coons* (1963) and *O Brien & Coons* (1963) which indicate that different cell populations respond to different antigens

There is copious evidence for the importance of cell proliferation in secondary antibody responses (*Vaughan et al* 1960 *O Brien & Coons* 1963 *Dutton* 1961 *Schooley* 1961 *Baney et al* 1962 *Nossal & Makela* 1962 *Sado & Makinodan* 1964) Although only a few mitotic figures were seen on the slides prepared from the cultures in the present study this still indicated a fair degree of cell proliferation We may therefore assume that the appearance of 19S antibodies in the culture medium primarily was a reflection of the proliferation of primed lymphoid cells The shortest doubling time for these cells was estimated to be

about 20 hours A continuous mobilization *in vitro* of unprimed cells is unlikely as we have not yet in this system been able to demonstrate a definite primary *in vitro* response

When increasing antigen multiplicities were used the lag phase of the resulting *in vitro* antibody responses was gradually shortened This could be due the increased chance for antigen to interact with a greater number of primed cells or/and to an increase in the mitotic rate of the lymphoid cells The highest antigen multiplicities (40 to 50) corresponding to about 40 to 50 μg viral protein used in the present study were apparently much too low to induce unresponsiveness in the cells

It is of interest to note that *in vitro* 19 S responses could be induced with an antigen multiplicity as low as 0 02 (0 02 μg virus protein) Since maximally 0 1 to 0 5 per cent of the spleen cells can be expected to be primed (*Landy et al* 1965) the ratio between viral particles and primed cells would be as low as 0 0001 This can be interpreted to suggest that a single hit with a receptor molecule on the cell surface is sufficient to induce the primed cells to renewed proliferation

Although some investigators (*Forbes & Turner* 1965 *Parenti et al* 1966 *Ripps & Hirschhorn* 1967) have reported that PHA stimulates immunoglobulin synthesis by lymphoid cells there are no unanimous results in regard to its effect on antibody formation Induction or elicitation of the antibody response was described by *Tao* (1964) and *Halliday & Garvey* (1966) while *Harris & Littleton* (1966) observed no effect and *Elves* (1967) and *Jennings & Oates* (1967) even reported a suppression of the antibody response The discrepancy between these results can partially be due to differences in the timing of the addition of PHA and in the physico chemical properties of the antigen used In the present study the continuous use of a medium containing PHA caused a distinct elevation of the antibody titres and increase in blastogenesis Restimulation *in vitro* with viral antigen evoked blastogenesis and a simultaneous or nearly simultaneous 19 S antibody response Thus under certain experimental conditions antigen and PHA are each alone capable of inducing both elevated antibody formation and blastogenesis When used simultaneously poliovirus and PHA had additive effects on antibody titres and degree of blastogenesis in the present *in vitro* system These results are compatible with the suggestion that the antigen stimulates the primed cells while PHA acts as a general stimulant activating both primed and unprimed lymphoid cells

The capacity of PHA to agglutinate erythrocytes and lymphoid cells is not restricted to these cells We have observed the effect of PHA on human diploid lung cells and human conjunctiva cells in monolayer cultures (*Svehag & Holmstrom* 1967) The cells were rounded appeared in clusters and had little tendency to spread out on the glass during the first two days of cultivation When the PHA was removed the cells gradually regained normal features and spread out on the glass surface

The spontaneous revival of the antibody producing capacity of lymphoid cells observed *in vitro* about 1 week after cultivation deserves special attention A weak antibody formation without intentional stimulation with antigen *in vitro* was also noted by *O Brien & Coons* (1963) *Halliday & Garvey* (1964) and *Bussard & Hannoun* (1966) The latter authors speculated about a possible spontaneous derepression of the immunological potentiality of a tissue when under culture conditions As in their system antibody formation was measured after incubation of the cells *in vitro* overnight with antigen (erythrocytes) an *in vitro* booster effect can not be excluded Why this effect only was obtained with cells which had been cultivated for a week remains to be explained In the present system the spontaneous transient *in vitro* 19S response could also be the result of antigenic stimulation associated with a release of virus antigen during the first days of cultivation It is considered unlikely that the spontaneous *in vitro* 19S response was evoked by stimulation with a medium component which crossreacted with one of the two known PV antigens

The cytological criteria for blastogenesis were based on the size of the cells and on the appearance of the nuclear chromatin and the nucleoli (*Fagraeus* 1960) Extremely few or no blastoid cells were observed in the cell suspension prior to *in vitro* cultivation Restimulation with poliovirus or cultivation of the cells in the presence of PHA caused blastic evolution which was even more pronounced when PHA was used A certain degree of blastogenesis was seen even when no polio virus or PHA was added to the medium Plausible explanations for this "background blastic evolution were stimulation by viral antigen released from damaged cells or by the calf serum or antibiotics present in the medium It could be shown however that some blastogenesis occurred also when cells from unsensitized animals were nourished by a medium devoid of antibiotics PHA and viral antigen This low degree of blastogenesis was seen also when autologous serum was used *Sabesin* (1965) similarly noted spontaneous blastogenesis when autologous serum without antibiotics was used

In the early phase of this work the blastoid cells observed were similar to the "transformed cells commonly observed after stimulation with PHA These cells occurred in small clusters were only 2 to 3 times the size of small lymphocytes and had a large nucleus with a fine fibrillary chromatin pattern Parallel to the increase in pyroninophilic blastoid cells there was a decrease in the number of small lymphocytes This finding is compatible with the concept that the blastoid cells have a lymphocytic origin (*Robbins* 1964) This conception is supported by the fact that the histochemical staining properties of blastoid cells are similar to that of lymphoid cells (*Quaglino et al* 1962) In the latter phase of the work where Parker 199 was substituted for Eagle in Earle s buffer as medium larger pyroninophilic cells with excentric ovoid nuclei were observed (Fig 10) These cells were 3 to 6 times the

size of small lymphocytes With time of cultivation their nuclei assumed an inactive appearance and the cytoplasma became vacuolated They resembled the blastoid cells described by *Sabesin* (1965) and *Hannoun & Bussard* (1966) in their *in vitro* studies of rabbit lymphoid cells In the present study these large cells often had small lymphocytes fixed to the cell surface (Fig 11) but phagocytosis of the lymphocytes or of india ink was not noted

SUMMARY

Cultures of isolated rabbit spleen cells from sensitized animals responded to antigenic (poliovirus) restimulation with secondary antibody response of up to 2 months duration The kinetics of the responses and the molecular type of antibody formed depended upon the antigen multiplicity (antigen/cell ratio) used for restimulation Low antigen multiplicities induced transient 19 S antibody responses with peaks around day 10 while higher antigen doses evoked also 7 S responses with peaks about 3 weeks after stimulation The *in vitro* antibody responses were potentiated when phytohaemagglutinin was incorporated into the medium Passively transferred 7 S antibody interferred with the induction of specific *in vitro* antibody responses Prior stimulation of the cells with heterologous antigen had no such effect

Viral antigen and phytohaemagglutinin were each alone capable of inducing both elevated antibody formation and blastogenesis A low degree of blastogenesis occurred even when unprimed cells were nourished by a medium devoid of viral antigen phytohaemagglutinin and antibiotics

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THE OCCURRENCE OF INTERFERON IN THE CEREBROSPINAL FLUID IN PATIENTS WITH BACTERIAL MENINGITIS

By

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Received 4 x 67

*It was originally assumed that interferon was formed exclusively in response to viral infection of animal cells (11) but during recent years it has been realized that interferon like inhibitors may also be formed or released under the influence of rickettsiae (10) toxoplasmas (19) and certain bacteria (20 2a) In addition certain cell extracts such as bacterial endotoxin (20) yeast polysaccharides (13 18) and phyto haemagglutinin (24) have proved to be able to stimulate a similar interferon induction

Interferon induction *in vivo* by bacteria was first demonstrated experimentally by *Youngner & Stinebring* (25) after intravenous in jection of large doses of virulent *Brucella abortus* cultures in chickens Later *Stinebring & Youngner* (20) observed that intravenous injection of *Serratia marcescens Salmonella typhimurium* and bacterial endo toxin induced interferon in mice At the same time *Ho* (8) showed that heat killed *Escherichia coli* could induce interferon like substances in the serum of rabbits

Interferon production has repeatedly been demonstrated in man in relation to viral infections (3 22 23 5 16 14) In a study of the cerebrospinal fluid (CSF) in 25 patients with bacterial meningitis *Gresser & Naficy* (5) revealed interferon like substances in three cases Two of these were children with *Haemophilus influenzae* meningitis while the third patient suffered from *Listeria* meningitis In addition he found interferon like substances in the CSF of a patient with *Kleb siella* pneumonia The patient showed clinical signs of encephalitis but normal CSF findings

Later *Michaelis et al* (16) found interferon like substances in the

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serum from two among five children with *H influenzae* meningitis Examination for interferon in the spinal fluids was not reported Bacteriaemia was also present in the two patients concerned It was emphasized that the interferon like substances in both cases were disclosed after the institution of antibiotic therapy *Larke* (14) has recently reported that he in a study of CSF from 44 patients with bacterial meningitis found interferon in two cases In one of the patients CSF *cultures yielded H Influenzae In the second case no micro organisms* could be demonstrated but the meningitis was presumed to be bacterial in nature because of a relatively large number of polymorphonuclear leucocytes and a low content of sugar in the CSF

Finally with a view to demonstrating the presence of interferon *Wheelock* (23) studied serum from 29 patients with acute bacterial infections but it was not present in detectable amounts in any of these cases

Judging from these investigations interferon production in response to bacterial infections seems to be relatively rare in man As however interferon can often be demonstrated in animals experimentally infected with a considerable number of different bacterial species it seemed to be of interest to perform further studies on the occurrence of interferon in bacterial infections in man The results of such a study of the CSF from patients with bacterial meningitis are reported below

MATERIALS AND METHODS

Viruses

Semliki forest disease (SFD) strain Smithburn was originally obtained from Casals in 1961 In this laboratory 6 passages were made by intracerebral inoculation into albino mice Stocks of SFD virus were prepared from infected mouse brains On the development of symptoms of encephalitis usually after two or three days the brains were removed after preceding exsanguination and ground with sand in 10 parts of PBS (pH 7 4) After centrifugation at 6000 r p m for 30 minutes volumens of 1 ml of the supernatant were stored in 5 ml ampoules at —70 C.

West Nile virus (WN) strain Egypt 101 was originally obtained from Casals in 1961 In this laboratory 6 passages were made by intracerebral Inoculation into albino mice The virus was prepared as described above under SFD virus

Media For all cell cultures was used Eagle s Basal Medium (CIBCO) to which were added penicillin 20 000 I U streptomycin 20 mg and glutamine 29 mg/100 ml Growth medium for human fibroblasts was supplemented with 20 per cent human serum during the first three passages and with 20 per cent inactivated calf serum during later passages To the growth medium for mouse embryo cells was added 15 per cent inactivated calf serum All maintenance media contained 2 per cent inactivated calf serum All media were adjusted to pH 7 3 by addition of a 2 8 per cent Na bicarbonate solution

Tissue Cultures

Human fibroblasts were prepared from skin biopsies by a method described by *Thelkelsen* (1) The cell cultures were kindly placed at m di po al by Dr Therkelsen in their 3rd–4th passage and used in the laborato y in the 4th 10th pass ges The grow th medium as Eagle s Basal Medium ith 0 per cent inact ated calf serum

Mouse embryo cells (ME) were prepared from 18 1 0 d y old emb from albino mice The embryos were removed under th n c the ia l y (a a n s e tion and at once placed in pre warmed PBS Aft em al of the le d ta l legs

and guts the embryonic parts were minced by knives washed in PBS and then treated with a trypsin solution (0 01 per cent Trypure Novo) under agitation by a magnetic stirrer for four or five periods of 10 minutes The first suspension of cells was discarded because of an ample content of erythrocytes Subsequent suspensions were pooled and the cells centrifuged at 800 r p m for 10 minutes The supernatant was decanted and the cells suspended in the growth medium with 4 × 10 cells to 150 ml of medium and stored in 1 litre Roux flasks which were incubated at 36 C The growth medium was Eagle s Basal medium with 15 per cent inactivated calf serum

Human Control Interferon

Control interferon was produced in human fibroblasts grown in 1 litre Roux flasks When the cells had grown to confluence the growth medium was removed and the cultures inoculated with WN virus (approx 10 I D per cell as determined by intracerebral inoculation into baby mice) The cultures were placed at 37 C for 1 hour with frequent gentle shaking to permit virus adsorption Maintenance medium 80 ml per flask was then added after which the cultures were incubated at 36 C for 72 hours The medium was then removed and transferred to dialysis tubing (Visking 10 mm in diameter) The tubing was immersed in Sørensen buffer pH 2 for 48 hours After dialysis to pH 7 4 the interferon content of the fluid was usually found to vary from 25 to 30 units per ml

Intracerebral inoculation of the fluid into baby mice did not result in disease or mortality indicating that interference was not caused by live virus

Cerebrospinal Fluids

All samples of CSF studied originated from patients with bacterial meningitis These samples had been submitted to the Diagnostic department of the State Serum Institute Copenhagen where the bacteriological diagnoses had been made The samples had been received from hospitals from all parts of the country The time which had elapsed from the withdrawal of the samples to the arrival at the Diagnostic Department varied from 2 to 8 hours During the transport the temperature of the samples must be presumed to have been 20–25 C On arrival the samples were stored at 4 C for 1–3 months and some of them then at —20 C for another 2 6 months The samples of CSF were centrifuged at 6000 r p m for 30 minutes and the supernatant was then used for the interferon assay

Clinical Data

Whenever possible clinical data were collected from the departments concerned through the loan of the hospital records but in some cases the information had to be taken from copies of the data submitted to the patient s physician on discharge from hospital

Interferon Assay

The interferon assay in homologous cells was made by the plaque inhibition method in cultures of human fibroblasts The cultures were grown in 50 mm plastic Petri dishes (Nunc Roskilde) with 0 75 × 10⁴–1 0 × 10⁴ cells in 5 ml of growth medium in each dish Throughout the experiment incubation was carried out at 37 C in a 5 per cent CO atmosphere On growth to confluence usually after 48 hours the medium was decanted and replaced by 1 ml of a 1 4 dilution of CSF in maintenance medium In most cases the assay was performed only in this dilution but occasionally when sufficient amounts of CSF were available two four or five fold serial dilutions were made Each dilution was tested in two culture After incubation for 5 hours the CSF was decanted and 0 5 ml of SFD virus containing 50 100 PFU was added to each culture After incubation for another hour the virus suspension was removed and 5 ml of 0 F per cent agar in Eagle s Basal Medium was added Shortly before use 10 mg of DEAE per 100 ml agar solution was added The agar was applied by the dishes by means of an automatic syringe When the agar had solidified the dishes were again placed in a CO incubator at 37 C After 40 hours staining with neutral red (125 μg per 11 h) was performed Plaque counts could then be made in the course of 8 10 hours

The interferon assay was considered to be positive when at least a 50 per cent reduction in the number of plaques in a 1 4 dilution of CSF had occurred In int r

feron titration the titre was expressed as the reciprocal of the dilution which showed a 50 per cent reduction in the number of plaques

Interferon assay in heterologous cells was performed on secondary cultures of ME cells After trypsinization the cells were removed from flasks with primary cultures of ME cells and placed in 50 mm plastic Petri dishes Otherwise the method was as described for interferon assay in human fibroblasts The agar for the ME cells contained 5 mg of DEAE per 100 ml

Virus Titration

The plaque method was used in secondary cultures of ME cells for the virus titration Petri dishes and medium as described above After growth to confluence the growth medium was decanted and 0 5 ml of 10 fold serial dilutions was inoculated each dilution was tested in 2 cultures The cultures were incubated for 1 hour following which the virus suspension was decanted and the agar solution applied as described above Staining with neutral red (1?5 μg per dish) was performed after 4? hours followed by plaque counting

RESULTS

A virus inhibitor was demonstrated in 33 out of 61 samples of CSF or in 28 out of 53 patients with bacterial meningitis

The titre of the virus inhibitor was studied only in some cases Most frequently total plaque inhibition was observed in 1 4 dilutions of CSF In cases where it was possible to perform titration titres of up to 160 per ml were obtained

In order to characterize the virus inhibitor (15) it was examined for species specificity in a heterologous cell system and for direct antiviral activity In addition the effect of the following procedures was investigated

Centrifugation at 108 000 g for 3 hours
Treatment with trypsin (Trypure Novo) 0 5 mg per ml CSF at 37 C for 1 hour
Treatment with ribonuclease 0 5 mg per ml CSF at 37 C for 1 hour
Heat treatment at 56 C for ½ hour
Dialysis at pH 2 and 4 C for 24 hours

As in many cases only small amounts of CSF were available all the above procedures could not be used on all the CSF samples revealing an antiviral effect A few of the samples were tested only for antiviral activity but in the majority of cases two or more of the procedures were applied Three samples of CSF were tested by all the methods In the cases where titration of the CSF was not performed any change in the antiviral activity due to the above procedures was assessed on the basis of the plaque inhibition percentage in a 1 4 dilution In all cases the individual procedures and tests gave the same results in the various samples of CSF

The inhibitory effect did not change after ultracentrifugation or ribonuclease treatment Exposure to trypsin completely destroyed the antiviral activity while heat treatment and dialysis resulted in partial loss of this activity Testing on mouse embryo cells showed no signs

of virus inhibition On incubation together with SFD virus for 1 hour prior to plaque titration on mouse embryo cells the CSF showed no direct antiviral action

The study thus showed that the antiviral factor found in the spinal fluids has characteristics which are compatible with human interferon (2) In previous studies (5 14) interferon in CSF has exhibited the same properties

The causative agents of the bacterial meningitides from which CSF was studied are listed in Table 1 The number of interferon positive CSF samples is related to that of studied samples for each bacterial species Similarly the number of patients with interferon in their CSF are related to the number of patients studied

TABLE 1

Proportion of Interferon positive CSF Samples and Patients Arranged According to Bacteria Isolated

Bacteria isolated	Number of CSF samples Positive/ total	Number of patients Positive total
Hæmophilus influenzae	7/14	7/14
Klebsiella	7/8	2/3
Pseudomonas	2/2	2/2
Escherichia coli	1/1	1/1
Proteus mirabilis	1/1	1/1
Citrobacter intermedium	0/1	0/1
Mixed infection with large Gram negative rods	5/5	5/5
Neisseria meningitidis	3 10	3/10
Diplococcus pneumoniae	2/6	2/6
Streptococcus	2/5	2/5
Staphylococcus	1/6	1/3
Listeria monocytogenes	2/2	2/2
Total	33/61	28/53

As some of the CSF samples originated from patients admitted to neurosurgical departments the bacterial spectrum differs to some extent from what is normally seen in spontaneous bacterial meningitides (12)

On the basis of the clinical data available it was not possible to assess whether the interferon content of the CSF bore any relation to the severity of the disease

Six cases terminated fatally subsequent to the withdrawal of CSF in four of these interferon was demonstrated in the CSF In another four fatal cases CSF was secured at autopsy and all four samples contained interferon

The pathogenic micro organisms in the fatal cases were as follows *H influenzae* and *N meningitides* in one case each *Listeria monocyto*

genes in two cases and large Gram negative rods in the remaining cases including a few in which a mixed infection was present

In some cases the CSF samples originated from patients in whom the meningitis was a complication of another disease or was referable to a diagnostic procedure Table 2 shows the various conditions which were complicated by meningitis and the causal bacterial species

The time elapsing from the onset of meningitis to the withdrawal of CSF might be presumed to be of great importance for the possibility of demonstrating interferon in the CSF In many cases it was difficult with certainty to ascertain the exact time of onset of the disease but in 43 patients it could be decided whether the duration of the disease was either up to or more than 24 hours The result of this analysis is shown in Table 3

TABLE 2

Demonstration of Interferon in CSF Samples of Cases of Meningitis Secondary to Another Disease Surgical or Diagnostic Intervention

	Number of patients Positive/total
Skull fracture with meningitis	4/5
Staphylococcus albus	1/1
Streptococcus	0/1
Pseudomonas	2/[illegible]
Klebsiella Staphylococcus albus	1/1
Cerebral tumour with postoperative meningitis	[illegible]/2
Klebsiella	2/2
Otitis media with meningitis	4/7
Diplococcus pneumoniae	2/4
Hæmophilus influenzae	1/2
Staphylococcus albus F coli Proteus	1/1
Malignant systemic disease with meningitis	4/4
Listeria monocytogenes	[illegible]/[illegible]
Proteus mirabilis	1/1
F alkalescens Klebsiella	1/1
Meningitis following myelography	2/2
Streptococcus	2/2

TABLE 3

Presence of Interferon in CSF Samples Collected During Early and Later Stages of the Disease

Duration of disease prior to lumbar puncture	Patients with interferon in CSF Positive/total	%
24 hours or less	1 19	63
More than [illegible]4 hours	11 4	46
Total	23 43	53

of virus inhibition On incubation together with SFD virus for 1 hour prior to plaque titration on mouse embryo cells the CSF showed no direct antiviral action

The study thus showed that the antiviral factor found in the spinal fluids has characteristics which are compatible with human interferon (2) In previous studies (5 14) interferon in CSF has exhibited the same properties

The causative agents of the bacterial meningitides from which CSF was studied are listed in Table 1 The number of interferon positive CSF samples is related to that of studied samples for each bacterial species Similarly the number of patients with interferon in their CSF are related to the number of patients studied

TABLE 1

Proportion of Interferon positive CSF Samples and Patients Arranged According to Bacteria Isolated

Bacteria isolated	Number of CSF samples Positive/ total	Number of patients Positive/ total
Hæmophilus influenzae	7/14	7/14
Klebsiella	7/8	2/3
Pseudomonas	2/2	2/2
Escherichia coli	1/1	1/1
Proteus mirabilis	1/1	1/1
Citrobacter intermedium	0/1	0/1
Mixed infection with large Gram negative rods	5/5	5/5
Neisseria meningitidis	3/10	3/10
Diplococcus pneumoniae	2/6	2/6
Streptococcus	2 5	2/5
Staphylococcus	1/6	1/3
Listeria monocytogenes	2/?	?/2
Total	33/61	28/53

As some of the CSF samples originated from patients admitted to neurosurgical departments the bacterial spectrum differs to some extent from what is normally seen in spontaneous bacterial meningitides (12)

On the basis of the clinical data available it was not possible to assess whether the interferon content of the CSF bore any relation to the severity of the disease

Six cases terminated fatally subsequent to the withdrawal of CSF in four of these interferon was demonstrated in the CSF In another four fatal cases CSF was secured at autopsy and all four samples contained interferon

The pathogenic micro organisms in the fatal cases were as follows *H influenzae* and *N meningitides* in one case each *Listeria monocyto*

DISCUSSION

The investigation has shown that interferon in the CSF occurs with a surprisingly high frequency in patients with bacterial meningitis. In the series studied interferon was demonstrated in more than one half of the cases, i.e. the frequency is as high or even higher than the percentages which have previously been reported in studies of CSF from patients with viral meningitis (5, 14).

The fact that interferon in the CSF from patients with bacterial meningitis was demonstrated with a considerably higher frequency in the present study than in previous ones may perhaps be explained on the basis of differences in the sensitivity of the test systems used. The system used in this study appeared to be relatively sensitive as compared with those in which other human cells and other viruses have been employed (6). The difference in frequency may also be due in part to the composition of the series of patients studied.

It has appeared that the range of different bacterial species which are capable of inducing interferon is considerably wider than has so far been demonstrated in animal experiments and clinical studies.

The study has confirmed previous reports on interferon production in response to infection by *H. influenzae*. It does not seem surprising that large Gram negative rods—possibly through release of endotoxin as has been seen in animal experiments (8, 20)—may induce interferon in the human organism. Interferon induction caused by meningococci has not previously been described but it may be supposed that it is likewise referable to affection of the cells by released endotoxin. More surprising is perhaps the interferon induction caused by Gram positive bacteria although this has previously been observed in a few cases. Thus *Gresser & Naficy* (5) found interferon in the CSF of a cortisone treated patient with agammaglobulinaemia and *Listeria* meningitis and Hann in a personal communication to *Ho* (7) described interferon induction in mice by pneumococci. *In vitro* experiments have also shown antiviral activity of certain Gram positive bacteria or substances liberated from them (1, 4) and it has been postulated that interferon production might be the cause of this activity (9).

In the present series there seemed to be an increased tendency to interferon induction when the meningitis occurred as a complication to a debilitating disease. The cause of this may be the bacterial aetiology which may be different in cases of secondary meningitis.

The possibility of the occurrence of an accompanying viral infection cannot be ruled out entirely in all cases. In particular in the cases in which the bacterial meningitis occurred as a complication to a malignant systemic disease the possibility of interferon production caused by a re-activated or acquired viral infection must be considered (17). However none of the cases revealed direct signs of such a viral infection. No attempts were made to isolate virus from the CSF because the

storage conditions had in all cases been unfavourable for the survival of a virus if present. In the two cases in which the meningitis was secondary to myelography admixture of virus did not seem likely. The symptoms were as in a bacterial meningitis and the patients responded promptly to antibiotic therapy. It also seems unlikely that interferon production can be induced by the chemicals used for the myelography (Conturex® and Per Abrodil©) but this will be subjected to a closer study in animal experiments. Generally speaking a fortuitous concomitant viral infection can scarcely be an essential cause of the frequent occurrence of interferon in the present series.

It is difficult to say to what extent daily CSF examinations throughout the course of the disease if practicable would have influenced the results. The time at which the CSF was studied during the course varied and interferon was demonstrated equally frequent a few hours after the clinical onset of the disease and several days later. In the two cases in which several samples of CSF were studied interferon was demonstrated throughout four days in one while it was present only on one occasion in the second case.

Michaelis et al. (16) found interferon in the serum over a period of five days in one of their patients with *H. influenzae* sepsis while another patient had interferon in the serum only on one occasion about seven days after the onset of the disease. The latter patient was studied on the day of admission and then one, four and 25 days later. In a few cases of bacterial meningitis *Gresser & Naficy* (5) and *Larke* (14) studied the CSF on several occasions with varying results. For example one patient (5) with *H. influenzae* had interferon in the CSF two days after the onset of the disease but not on the third day. Two other patients (14) had interferon in the CSF on the first and first—third days of disease respectively but then the interferon disappeared. In animal experiments it was usually possible to demonstrate interferon 1–2 hours after the injection of bacteria or endotoxin and in most cases the concentration reached a maximum after 2–12 hours; the interferon then disappeared within the next 24–48 hours (20). However such animal experiments in which a massive dose of bacteria or endotoxin is injected resulting in an intense affection of the cells can scarcely be compared with a naturally occurring bacterial infection in the human organism.

It would seem reasonable to assume that more frequent CSF examinations would have revealed interferon in an even larger number of patients with bacterial meningitis.

As the interferon production induced by bacteria is assumed to be due to an affection of the cells by the bacterial breakdown products and as the disintegration of bacteria must be expected to increase steeply after the institution of adequate antibiotic therapy, it has been claimed that this therapy may stimulate the interferon production. This assumption was supported by the observation by *Michaelis et al.* (16)

who did not find interferon in the serum until after the institution of antibiotic therapy However in many of the patients in the present series interferon was revealed in the CSF before they received antibiotics Accordingly no significant relationship seems to exist between such treatment and the occurrence of interferon

The observed lack of correlation between the number of leucocytes and the presence of interferon in the CSF is in agreement with observations made in previous studies (5 14)

SUMMARY

Sixty one samples of CSF from 53 patients with bacterial meningitis were studied for antiviral activity A virus inhibitor whose characteristics were compatible with interferon was disclosed in 33 samples of CSF originating from 28 patients Interferon was demonstrated in association with infections caused by widely different bacterial species Gram positive as well as Gram negative The occurrence of interferon was not with certainty related to the commencement of antibiotic therapy and there was no correlation between the presence of interferon and the number of leucocytes

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RUBLLLA VIRUS NEUTRALIZATION IN HEATED SERA

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In virus laboratories the commonly used procedure of heating sera to 56 C for 30 minutes preceding titration of antibody has been reported to reduce titres of neutralizing antibody to rubella virus (10 12 13) In a previous study (7) inconsistent results were obtained in rubella neutralization tests when heated (56 C for 30 minutes) and unheated human sera were tested simultaneously Usually neutralizing antibody to rubella virus could be demonstrated only in unheated sera However the addition of fresh normal serum has been shown to restore the neutralizing activity of heated serum and also to enhance rubella antibody titres (10 13 14) The studies reported here were undertaken to further investigate this effect in order to establish a suitable method for titration of rubella neutralizing antibody in heated human sera

MATERIALS AND METHODS

Tissue culture Monolayer cultures in roller tubes were prepared from the rabbit cornea cell line SIRC (6) The tubes were seeded with 200 000 cells contained in 1 2 ml of growth medium and incubated in stationary racks at 37 C during the 4 5 days outgrowth period

Media The cells were grown in Earle s solution supplemented with 10 per cent inactivated calf serum and containing per litre lactalbumin hydrolysate 1 7 g yeast extract Difco 0 57 g and sodium bicarbonate 0 84 g As maintenance medium was used Eagle s minimum essential medium supplemented with 1 per cent inactivated calf serum and containing per litre l glutamine 0 292 g and sodium bicarbonate 2 24 g Both media contained per litre 0 05 g streptomycin sulphate and 0 01 g neomycin sulphate

Virus Rubella virus strain Judith was used in the form of tissue culture fluid in its 7th SIRC passage titering approximately 6 log 10 TCID50 per ml

Titration of virus Virus titrations were performed using ten fold dilutions in maintenance medium Five roller tube cultures per dilution and an inoculum of 0 2 ml were used

Titration of antibody Antibody titrations were performed employing serial two fold dilutions of sera in the maintenance medium An amount of 0 5 ml of each serum dilution was mixed with 0 5 ml of virus dilution containing approximately 100 TCID 0 per 0 1 ml After incubation at 34 C for one hour 0 2 ml of the serum virus mixture were inoculated into each of two tube cultures containing 1 8 ml of maintenance medium The tubes were rotated at 34 C and examined for cytopathic

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effect on the seventh day after inoculation The rubella neutralizing antibody titre was calculated by the method of Karber and expressed as the reciprocal of the initial serum dilution in the serum virus inoculum *A titre less than 4 is recorded as 0* throughout this paper

Inactivation of sera Human and animal sera were employed Sera inactivated in a water bath at 56 C for 30 minutes are referred to as heated sera All sera heated as well as unheated were stored at minus 20 C

Human sera Human sera used in the experiments were collected at random from blood donors or they were derived from patients suspect of rubella infection

Animal sera Guinea pig rabbit and horse sera were prepared from blood obtained from the animal farm of this institute while calf serum was prepared from blood collected at the municipal abattoir of Copenhagen

Determination of the haemolytic activity of the sera was performed according to a routine procedure using the highest serum dilution showing complete haemolysis as the end point The haemolytic titres of the employed animal sera were as follows Guinea pig serum 60 rabbit serum 10 calf serum 0 horse serum 0

The animal sera used in the experiments demonstrated no inhibitory effect to rubella virus

In experiments where the effect of animal sera was studied these sera were added to the medium used as diluent for the preparation of serum and virus dilutions The amount of animal serum added is expressed as per cent animal serum in the diluent medium

EXPERIMENTS AND RESULTS

Inhibitor Experiment

In order to determine whether the reduction of rubella neutralizing antibody in heated human sera could arise from a possible inhibitor occurring in the serum as a result of the process of heating the following experiment was carried out Unheated positive (antibody containing) human serum was mixed with equal parts of heated and unheated negative (antibody free) human serum respectively and neutralization tests were performed on these mixtures The result of the experiment which is recorded in Table 1 shows identical titres whether the positive serum is mixed with heated or unheated negative serum and does accordingly not support the idea of any production or release of an inhibitor to rubella neutralizing antibody by heating of human serum

TABLE 1

Rubella Neutralizing Antibody Titres of Unheated Positive Human Serum Mixed with Heated or Unheated Negative Human Serum

Serum	Antibody titre
Negative serum unheated	0
Negative serum heated	0
Positive serum unheated	32
Positive serum unheated mixed with negative serum heated	32
Positive serum unheated mixed with negative serum unheated	23

The Effect of Various Animal Sera on Rubella Neutralization

A potentiating effect of guinea pig serum on rubella neutralizing antibody has recently been described by *Rawls Desmyter & Melnick*

(14) *McCarthy & Taylor Robinson* report a similar effect of rabbit serum which in their experience is superior to guinea pig serum (11)

The following study was carried out in order to determine the effect of various animal sera in neutralization tests with rubella virus

Heated and unheated human sera were used with the addition of 5 or 10 per cent unheated serum from guinea pig rabbit calf or horse The results are summarized in Table 2

TABLE 2

The Effect of Varying Amounts of Animal Sera in Rubella Neutralization Test

Human serum		without addition	Per cent unheated serum from							
			Guinea Pig		Rabbit		Calf		Horse	
			5	10	5	10	5	10	5	10
A	Unheated	8	16	8	16	23	8	11	11	11
	Heated	0	23	11	4	4	0	0	0	4
B	Unheated	23	45	16	32	UR	16	8	23	45
	Heated	0	32	45	11	UR	0	0	0	90
C	Unheated	64	45	45	UR	UR	45	90	32	64
	Heated	32	23	45	UR	UR	0	6	11	16

UR = Unreadable

It can be seen that the reduction in neutralizing antibody titre in heated human sera is most effectively compensated for by the addition of guinea pig serum 5 and 10 per cent serum having the same effect

Results obtained with the rabbit serum also suggest an ability of this serum to restore antibody titres in heated sera In our hands however the effect of rabbit serum is rather inconsistent and because of an irregular occurrence of viral cytopathic effect in the tissue cultures interpretation of the neutralization tests has often been found impossible

It has not been possible to demonstrate any toxicity of rabbit serum to the cells

Calf serum does not appear to be able to restore the neutralizing rubella antibody titre of heated sera With one of the heated sera (Serum C) it even appeared to cause a reduction in the neutralizing titre

Addition of 10 per cent horse serum shows an effect which equals that of guinea pig serum while a 5 per cent concentration is unable to restore the titre of heated sera

In order to unveil a possible influence of the animal sera on the virus titre control virus titrations were in each experiment performed with and without admixture of these sera The addition of animal sera to neutralization tests was in all experiments found not to influence the titre of the virus

Reading the neutralization tests virus break through was occasionally

seen in the lower serum dilutions as has been noted also by several other workers (10 13)

Amount of Guinea Pig Serum Required for Enhancement of Activity of Heated Sera

The results of previous experiments indicated that among the various animal sera tested guinea pig serum was the most effective It was accordingly decided to investigate this serum further

The amount of guinea pig serum necessary for enhancement of the neutralizing activity of heated sera was determined using concentrations of 2 1 0 5 0 25 0 12 0 06 and 0 03 per cent Preliminary experiments had indicated that concentrations of 3 and 4 per cent did not differ from the results obtained with 5 and 10 per cent guinea pig serum

The experiments were repeated and the results of a representative test are shown in Table 3 It will be seen that all concentrations of guinea pig serum from 0 25 per cent to 2 per cent corresponding to 1 5 and 12 haemolytic units respectively are equally capable of enhancing the rubella neutralizing antibody titre in heated human serum while concentrations below 0 25 per cent do not exert any demonstrable effect

Based on these findings it was found practical to use a concentration of 1 to 2 per cent unheated guinea pig serum in future titrations of rubella neutralizing antibody

TABLE 3
The Effect of Varying Amounts of Guinea Pig Serum (GPS) in Rubella Neutralization Test

Human serum	Per cent unheated GPS							
	0	0 03	0 06	0 12	0 25	0 5	1	2
Unheated	32	32	45	23	45	45	90	64
Heated	11	16	16	23	128	90	90	90

The Effect of Heated and Unheated Guinea Pig Serum

The influence on rubella virus neutralization of heated and unheated guinea pig serum was studied using a 2 per cent concentration of guinea pig serum Both heated and unheated human sera were examined

The data summarized in Table 4 confirm that the procedure of heating human sera at 56 C for 30 minutes considerably reduces the rubella neutralizing antibody titres in some instances to such a degree that the antibody cannot be demonstrated

The titrations of heated human sera in the presence of unheated guinea pig serum show an enhancement of the antibody titres which

are raised to or above the level found in unheated human sera titrated without guinea pig serum In two of the tested sera the titres were found to increase 4 to 8 fold Comparing the titres of heated human sera obtained with and without the addition of unheated guinea pig serum the rise in titre is seen to be even greater with a maximal enhancement of 90 fold

TABLE 4

The Effect of 2 Per Cent Guinea Pig Serum (GPS) on Neutralization of Rubella Virus

Serum no	Heated human serum			Unheated human serum		
	without GPS	heated GPS	unheated GPS	without GPS	heated GPS	unheated GPS
1	0	0	8	16	11	23
2	4	6	32	16	16	128
3	0	0	90	23	32	128
4	8	8	64	23	23	128
5	0	0	32	32	16	32
6	4	4	360	45	32	180
7	6	6	128	64	64	180
8	11	16	45	64	64	90

In the unheated human sera a 4 to 8 fold rise in antibody titre can be seen in half of the sera tested The titre of one serum is unchanged and the apparant enhancement of titres in the rest of the sera is hardly significant

Heated guinea pig serum does not influence rubella neutralizing antibody titres of heated and unheated human sera

A number of known antibody free human sera was titrated heated as well as unheated with and without addition of heated and unheated guinea pig serum In none of these tests was rubella neutralizing antibody demonstrated

DISCUSSION

It is common practice in virus laboratories to heat human sera for 56 C for 30 minutes in order to make them suitable for complement fixation and haemagglutination tests The same procedure has also been found to eliminate or reduce the presence of non specific virus neutralizing antibodies

However for a number of viruses such as herpes variola and Western equine encephalitis this heat inactivation has been found to result in loss of specific neutralizing activity It has been shown that this activity can be restored by the addition of fresh normal serum (2 9 15)

Rubella virus neutralization has been shown to give erratic results if the sera have been heat inactivated (7 10 12 13)

The addition of fresh unheated normal guinea pig or rabbit to the serum virus mixtures enhances rubella virus neutrali-

heated sera (10 13 14) but several investigators prefer the use of unheated human sera rather than adding the extra variable of an animal serum to the test (1 4 5)

The data obtained in the present study suggest that unheated sera from three out of four animal species tested are able to restore the rubella neutralizing activity of heated human sera

The potentiating effect of 10 per cent guinea pig serum and 10 per cent horse serum was found to be of the same magnitude but while the latter serum did not demonstrate any effect in lower concentrations guinea pig serum gave consistent results in all concentrations down to 0 25 per cent

The use of rabbit serum gave inconsistent results with day to day variations as regards the ability of the serum to restore rubella antibody titres in heated human sera

Calf serum did not demonstrate any potentiating effect In one instance titrations with the addition of this serum even resulted in titres lower than those obtained without addition of animal serum

By use of normal guinea pig serum containing 10 haemolytic units of complement in neutralization tests *Rawls Desmyter* and *Melnick* observed a 4 fold to 16 fold enhancement of rubella neutralizing antibody titres in all of 8 sera tested (14)

The present study shows the same consistency in the potentiating effect of guinea pig serum Using 2 per cent guinea pig serum corresponding to approximately 12 haemolytic units in the serum and virus dilutions all of the 8 sera tested showed a rise in antibody titre of 4 fold or more the maximal enhancement being approximately 64 fold Considering that a two fold increase in titre implies a 100 per cent increase in antibody concentration it will be seen that at least half of the rubella antibody in heated serum is dependent on one or more normal serum components present in the guinea pig serum

When the same human sera were titrated without previous heating the influence of normal guinea pig serum appears to be less pronounced A rise in antibody titre equal to or greater than 4 fold was seen in half of the sera the maximal enhancement of 8 fold being observed in only one serum

From Table 4 it is apparent that the two heated sera without neutralizing capacity (Serum no 3 and 5) on addition of unheated guinea pig serum show titres of 90 and 32 respectively and two other heated sera (Serum no 2 and 6) show rises from 4 to 32 and 360 respectively Consequently a serum demonstrating a low titre in a titration without guinea pig serum does not necessarily also show a relatively low titre when titrated in the presence of guinea pig serum This fact might be explained by the existence of several types of rubella neutralizing antibodies with different requirements for a co factor and the presence of these antibodies in varying amounts in the individual sera depending on the time of collection after onset of illness (8)

It has not been possible in the present study to confirm the finding by *Rawls Desmyter & Melnick* that the haemolytic activity of guinea pig serum parallels its antibody potentiating action (14). In our experience the same effect is obtained regardless of the number of haemolytic units ranging from 1.5 to 12.

It has been clearly established that the components of normal serum which assist specific neutralization of rubella virus are heat labile (11, 14), a finding which is confirmed in the present study. The nature of the factor or factors responsible for the enhancement of rubella antibody is, however, not fully determined.

Rawls, Desmyter & Melnick suggest that the action may be due to complement since they found that removal of complement by a heterologous antigen antibody system also removed the rubella antibody enhancing effect (14).

However, the finding in the present study that 10 per cent horse serum exerts an effect similar to that of guinea pig serum indicates that the factor or factors involved cannot be full complement since horse serum is lacking the C 2 complement component (3).

In this study no production of inhibitory substances in the heated human sera was demonstrable.

SUMMARY AND CONCLUSION

Studies on the influence of sera from four animal species on titrations of rubella neutralizing antibody have shown a reliable potentiating effect of unheated normal guinea pig and horse serum. Guinea pig serum in concentrations of from 0.25 to 10 per cent in the medium used as diluent in the serum and virus dilutions were equally effective while more than 5 per cent horse serum was needed to show an effect.

Heated and unheated human sera were titrated with and without the admixture of guinea pig serum. In neutralization tests with heated human sera all of the 8 sera tested showed a rise in antibody titre of 4 fold or more, the maximal enhancement being 90 fold. When the same human sera were titrated without previous heating a rise in antibody titre equal to or greater than 4 fold was seen in half of the sera, the maximal rise being 8 fold.

Heated guinea pig serum did not have such properties. The study suggests, however, that the factor or factors responsible for the enhancement of rubella antibody titres is not full complement.

Unheated guinea pig serum in small amounts has thus been found capable of restoring the neutralizing capacity of heated human sera and also of enhancing the neutralizing effect of unheated sera.

Based on these findings it has been found practical to titrate rubella neutralizing antibody in heated human sera in the presence of guinea pig serum. A concentration of unheated guinea pig serum of 1 to 2 per cent in the medium used for dilution of serum and virus is suggested.

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STUDIES ON MICROCOCCAL PHAGES

Evidence of Phage Conversion without Signs of Lysogeny or Vegetative Multiplication of Phages in the Converted Bacteria

By

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In several bacteriophage host systems infection with so called converting phages has been shown to produce alterations in the properties of the bacteria In *Corynebacterium diphtheriae* the converting phage can control the production of exotoxin (*Freeman* 1951 *Barksdale & Pappenheimer* 1954 *Groman* 1955) in group A streptococci the production of erythrogenic toxin (*Zabriskie* 1964) In *Mycobacterium smegmatis* (*White et al* 1962) *Brucella abortus* (*Jones et al* 1962) and *Shigella dysenteria* (*Li et al* 1962) such infection has been shown to produce changes involving the synthesis of components of the cell wall with consequent change in the morphology of the bacteria or colonies In several strains of *Salmonella* groups E 1 and E 3 converting phages can initiate the synthesis of a new somatic antigen (*Uetake et al* 1955) Infection of *Staphylococcus aureus* of phage type 80/81 with phages of serogroup A may render the bacteria sensitive to phages of serogroup B (*Rountree* 1959 *Blair & Carr* 1961)

It has thus been shown that phages with converting capacity can exert influence on a variety of bacterial properties In group A streptococci (*Zabriskie* 1964) *Brucella abortus* (*Jones et al* 1962) and *Shigella dysenteriae* (*Li et al* 1962) the converting ability of the phage is manifested during vegetative multiplication

In *Staphylococcus aureus* (*Rountree* 1959 *Blair & Carr* 1961) the conversion is due to established lysogeny This also applies to *Salmonella anatum* In this bacterium the new phage determined antigen can be demonstrated almost immediately after the phage has penetrated into the bacteria (*Uetake et al* 1958)

In *Corynebacterium diphtheriae* the toxin production is believed to be related to vegetative phage multiplication (*Barksdale et al* 1961) but later investigations (*Miller et al* 1966) have failed to produce evidence in support of this view

Defective mutants of salmonella phage [illegible] that have lost their ability to multiply vegetatively can establish lysogeny and determine

the synthesis of new somatic antigen (*Uetake* 1959) Bacteria lysogenic with such phages are resistant to the homologous phage Demonstration of the relationship between the new antigen and the prophage requires knowledge of the history of the bacteria

This paper describes the changes in the properties of a *Micrococcal* strain after infection with phage without demonstrable vegetative phage multiplication or lysogeny in the changed bacteria

MATERIAL

Bacteria

A *Micrococcal* strain found on a plate inoculated with a *Staphylococcus albus* strain

The micrococci possess no oxidative fermentative properties for glucose galactose laevulose lactose sucrose rhamnose inosite dulcite mannitol sorbite xylose arabinose and adonitole The strain is strictly aerobic according to the taxonomic criteria for the genus Micrococcus (*Frans Bradford & Niven* 1955) After 18 hours incubation on solid medium at +30 C–+37 C the micrococci grow with the formation of yellow colonies 2–3 mm in diameter After further incubation for one day at room temperature the colonies become intensely yellow

Phage

The plate on which the micrococci were found was stored at room temperature for about one week During this time one of the micrococcal colonies became vitreous This type of change so called glassy degeneration is a sign of phage infection of a colony (*Twort* 1915)

The changed colony was suspended in broth After centrifugation the supernatant was passed through a membrane filter and titrated on micrococci taken from colonies without signs of phage infection Phages from one of the plaques obtained were propagated and this harvest was used as a basic stock The phage preparations used in this investigation were obtained on propagation phages from the base stock through one passage on the host strain (see page 287) The plaques obtained on titration in soft agar were 2 3 mm large distinct and clear without any demonstrable secondary growth The titres noted were 1–2 × 10 plaque forming units/ml The phage is here called *a* phage

Substrates

Tryptone Soya Agar and Tryptone Soya Broth (Oxoid)

Fermentation substrate Andrade Peptone Water (Oxoid) 15 g ager 10 g sugar 10 g distilled water 1 000 ml pH 7 2

METHODS

Aeration of broth cultures The bacteria were inoculated into 4 ml of broth i tubes 20 mm in inner diameter The tubes were shaken in a shaking apparatus The technique gave reproducible values for growth rate

Anaerobic culture was done in ... jars (Willis 1964) The ... gas consisted of 94 per cent hydrogen and 6 per cent carbon dioxide

Determination of the number of bacteria in broth cultures was done with ph t meter after calibration in a counting chamber

Titration and propagation of phage was done with the agar layer method ... 1959) Bottom layer Tryptone soya agar with 400 µg CaCl/ml The soft agar was stored in a waterbath at +45 C before use On titration 0 5 ml of broth with 4 5 × 10 bacteria in logarithmic growth phase ml was added The volume of the phage dilution was varied from 0 1 to 0 5 ml On propagation twice the amount of bacteria was added Phages were added until the ratio between bacteria and phage was 100 1 and the plates were incubated at +30 C over night and harvested the following day

Harvesting of phages 5 ml of broth was poured onto the plates the layer of ...

agar was cut into small pieces and transferred to tubes After centrifugation for 30 minutes at 2 000 *g* the supernatants were passed through a membrane filter (group 3 Gottingen) with a mean pore size of 300 mμ distributed among tubes and stored at +4 C

All dilution of phages and bacteria was done with broth

Treatment with DNAase Varidase® was added to the phage lysate to a final concentration of 200 units of streptodornase/ml (*Christensen* 1949) The mixture was incubated for 3 hours at +30 C

Precision of Phage Assay

On incubation of titration plates from the same dilution of a phage in +30 C and +37 C the number of plaques observed after incubation at +37 C was about 80 per cent of that found on incubation at +30 C The plaques that developed at +37 C were *on an average somewhat smaller and varied more widely in size than* those that developed at +30 C The incubation temperature used for titration of a phage was therefore +30 C Unless otherwise stated 5 titration plates were made for each dilution

Two criteria must be fulfilled to obtain a quantitatively reliable evaluation of the plaque count namely the *Poisson* distribution of the plaque number and the linearity of the relationship between the concentration of phage suspension and the plaque number (*Loren* 1962)

Table 1 gives the distribution of the plaques obtained on 50 titration plates with a phage dilution that gave on an average 2 20 plaque per plate The level of confidence for 7 classes is 11 1 for P ≈ 0 05 The agreement between the expected and *the observed distribution* was *good*

Titration plates were prepared with successive twofold dilutions of phage The correlation found between the concentration of the phage suspension and the number of plaques was linear (Fig 1)

The titration method thus permitted quantitative determination of the phage

Antiphage serum was prepared by repeated subcutaneous injection of about 5×10^{10} phages per injection without supplementary adjuvans into rabbits

Titration of antiphage serum was done according to *Adams* (1959) The K values obtained were about 10 Antiphage serum diluted 1 10 reduced the number of plaques by more than 99 9 per cent within 10 minutes

Passage in antiphage serum About 10^4 bacteria were inoculated in 2 ml broth *with antiphage serum 1 10 and incubated during aeration at +37 C for 8 hours* and then diluted and plated

Adsorption of phages 0 1 ml of phage dilution was added to 0 9 ml of broth with 400 μg $CaCl_2$/ml containing 5×10^9 centrifuged and resuspended bacteria/ml The mixture was incubated at +37 C in a waterbath for 30 minutes then diluted 1 100 in +4 C broth and centrifuged for 10 minutes at 2 000 *g* and the supernatants were

TABLE 1

Distribution of Plaques on Titration of a Phage

Number of plaques (i)	Observed number of plates with (i) plaques	Expected number of plates with (i) plaques
0	7	5 540
1	10	12 188
2	13	13 407
3	10	9 832
4	7	5 408
5	2	2 379
6	1 } 1	0 872 } 1 246
>6	0	0 374
Total	50	50 000

Mean 2 20 χ^2 1 3702

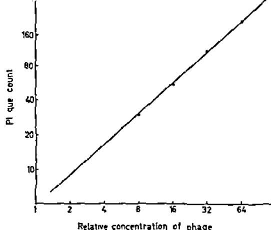


Fig 1

Relationship between concentration of phage and plaque number The plaque counts are plotted against the relative phage concentration both on a logarithmic scale

titrated 0 1 ml of phage dilution in two tubes with 0 9 ml of broth was incubated at +37 C and treated in the same way These two tubes served as controls

Demonstration of free phage in phage carrying clones The bacteria were incubated in aerated broth at +37 C to density 5×10^{8}–10^{9}/ml Soft agar plates were prepared with 0 5 ml of the broth and incubated at +30 C over night

The multi point inoculation method (Beech Carr & Codner 1955) was used in the examination of bacteria from a large number of colonies for ability to grow on soft agar containing phages

Control of sterility of phage suspension antiphage serum and CaCl solution was done in tryptone soya broth and an tryptone soya agar incubated at +37 C for 4 days

Isolation of a clone Since the micrococci in broth grow in clusters of up to 12 bacteria and since about 80 per cent of the clusters contain more than 4 bacteria the terminal dilution method could not be used for isolating a clone A reproducible

TABLE 2

Relation between Number of Counted Micrococci in Counting Chamber and Number of Colonies Developed

Mean number of bacteria counted	Mean number of colonies	Percentage of colonies of number of counted bacteria
2 4	1 3	54
2 1	0 8	38
1 4	0 6	42
1 5	0 6	40
1 [illegible]	0 5	41
1 3	0 5	38
1 2	0 5	41
2 9	1 5	52
1 5	0 7	47
1 [illegible]	0 8	47

The figures given for the number of counted bacteria and colonies developed are multiples of 10 Counting was done in a Burker counting chamber 10 or 0 C–D squares were counted

correlation between the number of micrococci counted in the counting chamber and the number of colonies that developed was however found when large plates were u ed (14 cm in diameter) and the inoculum was carefully spread with an angular glass rod When the number of colonies was less than about 150 per plate the average number of colonies on 10 plates was 38-54 per cent of the mean of the number of bacteria counted (*Table 2*)

Micrococci were passed three times in antiphage serum and inoculated on plates in the above mentioned manner after each passage This was done to eliminate any contaminating phages Micrococci from one of the colonies obtained was used as host strain in the investigation These micrococci derived on an averag from two bacteria and were regarded as a clone During the period of the investigation the strain was subcultured daily on tryptone soya agar plates

Infection of Micrococci with Phages on Solid Medium

Micrococci in logarithmic growth phase were centrifuged and resuspended to a density of 2-3 × 10⁸ bacteria /ml and about 1 ml was inoculated onto tryptone soya agar plates After the broth had dried 0 2 ml of phage dilution was dropped onto the plates which were immediately incubated at +37 C for 24 hours The secondary *grov th that developed in the areas with confluent lysis was transferred to broth* with a platinum loop Bacterial suspensions in suitable dilution were incubated without areation at +37 C for 60 minutes with antiphage serum 1 10 to eliminate free phages The bacteria were then spread on plates with a platinum loop containing about 0 01 ml The plates were incubated at +37 C for 24 hours and then stored for one day at room temperature before plates with well separated colonies were further investigated

Infection of Micrococci with Phages in Broth

0 5 ml of phage suspension was added to 0 5 ml of centrifuged and resuspended bacteria with a density of 5 × 10 /ml The mixture was incubated in a waterbath at +37 C for 30 minutes diluted 1 100 in +4 C broth for titration of unadsorbed phages and diluted in antiphage serum 1 10 The latent period was about 25 minutes after end of adsorption Dilutions in antiphage serum were kept at +37 C for 90 minutes in order to neutral ze e en newly developed phages and then in oculated in plates The plates were incubated at +37 C for 24 hours and then stored for one day at room temperature before the colonies were examined.

As control 0 5 ml of broth was added to 0 5 ml of the bacterial suspension and incubated at +37 C at the same time as the phage bacteria mixture The bacterial input was determined b plate count At time 30 minutes the bacteria were diluted in antiphage serum The bacteria in antiphage serum were kept at +37 C for 90 minutes and then spread on plates The dilution of antiphage serum 1 10 did not influence growth of the micrococci or colony count No growth of micrococci could be demonstrated in unaerated broth during at least three hours

400 µg CaCl /ml was added to the broth in adsorbtion tubes and all dilution tubes

RESULTS

Infection of Micrococci with Phages on Solid Substrates

To assess the approximate phage titre before titration in soft agar the titre was roughly estimated by dropping 0 1 ml of the dilutions on plates seeded with micrococci

It was found that considerable secondary growth appeared in areas with confluent lysis on plates incubated at +37 C for 24 hours to which phage dilution containing about 10⁹ p f u /ml was added When a phage suspension containing about 10⁵ p f u /ml was added little or no secondary growth appeared No demonstrable secondary growth appeared in the areas with confluent lysis on corresponding plates incubated at +30 C not even after several days incubation

TABLE 3

Production of Pigment by Bacteria Obtained from Secondary Growth after Infection with α Phage of Micrococci on Solid Substrates

Experiment	Number of colonies inspected	Number of yellow colonies	Number of white colonies	Percentage of white colonies
A	752	728	24	3 2
B	496	486	10	2 0
C	891	857	34	3 8
D	296	290	6	2 0
F	1084	1066	18	1 7

Secondary growth was suspended and diluted in broth and incubated without aeration at +37 C for 60 minutes in the presence of antiphage serum 1 10 About 0 01 ml of the suspension was spread on plates After incubation at +37 C for 24 hours and for a further day at room temperature plates with well separated colonies were examined

In experiment C D and E mutants of micrococci resistant to 12 μg oleandomycin/ml were infected In experiment D and F micrococci were infected with DNA ase treated phage lysate

Table 3 gives the results of inspection of colonies developing from secondary growth obtained after incubation at +37 C for 24 hours when phage suspension containing about 10^9 p f u /ml was added

Most of the colonies were of the same morphological appearance as those of uninfected micrococci and produced yellow pigment 2–4 per cent were of the same morphological appearance as these but produced white pigment

To distinguish micrococci not infected with phage from micrococci obtained from secondary growth the latter are referred to as My(α) (bacteria from yellow colonies) and Mw(α) (bacteria from white colonies)

To distinguish phage susceptible bacteria from lysogenized or selected phage resistant mutants My(α) and Mw(α) were inoculated on the surface of soft agar containing about 10 α phages/ml Control experiments showed that the host strain inoculated on such substrate gave no growth One might expect lysogenized micrococci or selected phage resistant mutants to grow irrespective of the presence of the phage

My(α) from 500 and Mw(α) from 92 colonies obtained in 5 experiments were examined

No bacteria grew on soft agar containing 10 α phages/ml They were thus still susceptible to the phage

About half of the colonies with My(α) as well as with Mw(α) contained free phage On soft agar plates prepared with 0 5 ml of broth culture with bacteria from such colonies 10^1 2×10^2 p f u /ml were obtained On soft agar plate prepared with 0 5 ml of supernatants from such broth cultures and with micrococci as indicator strain fewer plaques were usually obtained The phages could be eliminated by pas

TABLE 4

Plaque Counts on titration the α Phage on Micrococci on My(α) and Mw(α)

Micro cocci		My (α)				Mw (α)	
		Before first subculture				Before first subculture	
		p f u /ml	e o p			p f u /ml	e o p
210	From colony I	2 3	1 0	From colony	I	39	19
	II	?8	1 3		II	29	14
	III	?4	1 0		III	28	13
	IV	?3	1 0		IV	20	9
	V	1 6	0 7		V	29	14
		After first subculture				After first subculture	
		p f u /ml	e o p			p f u /ml	e o p
??1	From colony I	51	23	From colony	I	234	100
	II	38	17		II	?30	100
	III	30	14		III	??4	100
	IV	41	19		IV	246	100
	V	33	15		V	217	100
		After three passages in antiphageserum				After three passages in antiphageserum	
		p f u /ml	e o p			p f u /ml	e o p
16	From colony I	36	2?	From colony	I	177	100
	II	35	??		II	158	100
	III	39	24		III	169	100
	IV	44	27		IV	148	100
	V	39	?4		V	152	100

The figure given for the number of plaques are multiples of 10
Efficiency of plating gives number of plaques in per cent of number of plaques obtained on micrococci

sage in antiphage serum but rarely by subculture on plates Thus a continuous development of phage appeared to occur in a small number of bacteria in such phage carrying clones which developed colonies of normal appearance

Only exceptionally could signs of phage infection in the form of glassy degeneration be observed and then not before the plates had been allowed to stand for about a week at room temperature

α phage was adsorbed to My (α) and Mw (α) taken from colonies found not to bear the phage The α phage was also titrated on these bacteria Adsorption and titration were repeated after each of 3 sub cultures on plates and again after 3 passages in antiphage serum

The results obtained in the examination of Mv(α) and Mw (α) from each of the five colonies (colony I–V) obtained in one experiment are described below The results in the four other experiments were the same as those accounted for here

My (α) and Mw (α) showed a changed relation to the phage in that the number of plaques was lower on titration of phages on these bac teria than on micrococci (Table 4)

This new property was not stable but changed after the first sub

culture on solid medium and was afterwards not affected by further subculture or passages in antiphage serum

There was however a difference between My (α) and Mw (α) in the changed relation to the phage Before the first subculture the number of plaques on My (α) was about 1 per cent of that on micrococci After the first subculture 14–27 per cent were obtained (Table 4) The corresponding figure for Mw (α) before the first subculture was 9–19 per cent On subsequent titration on these bacteria the number of plaques was the same as that found on micrococci

The plaques that developed on My (α) and Mw (α) both before and after the relation to the phage become stable were of the same morphological appearance as those obtained on micrococci

The changed relation to the phage was probably not due to change in adsorption properties It is clear from Table 5 that no difference was found in rate of adsorption of phages to micrococci and to My (α) and Mw (α) before or after the relation to the phage had become stable (Table 5)

TABLE 5

Adsorption of a Phage to Micrococci Mw(α) and My(α)

Experiment 1 Control 196 p f u /ml		
	Micrococci	Mw (α) From colony I A
Unadsorbed phage	26 – 44	21 – 50
Mean	35	34
Percentage adsorbed phage	87 – 78	89 – 74
Mean	82	83
Experiment 2 Control 219 p f u /ml		
	Micrococci	My (α) From colony I-A
Unadsorbed phage	24 36	31 – 54
Mean	30	43
Percentage adsorbed phage	89 84	86 – 75
Mean	86	80

Input bacteria 5×10 /ml

Adsorption time 30 minutes at +37 C The mean values for unadsorbed phage are means from five adsorption tubes with My (α) and Mw (α) and from two absorption tubes with micrococci The above figures apply to My (α) and Mw (α) both before and after the first subculture and after passages in antiphage serum

No segregation of pigmentation or morphology of the My (α) and Mw (α) colonies was found on subculture on plates or passage in antiphage serum At each passage in antiphage serum 500–1000 colonies developed from each tube were inspected

Infection with a Phage in Broth

On infection of micrococci with α phage at a multiplicity of infection (m o i) 14–20 about 1 per cent of the bacteria survived

The number of surviving bacteria after infection of Mw (α) at m o i 10 was about 2 per cent against about 20 per cent after infection of My (α) at the same m o i The changed relation to the phage of My (α)

TABLE 6

Infection with α Phages in Broth

Bacteria	Input bacteria (photometer)	Input bacteria (colony count)	Input phages	Adsorbed phages	m o i	Survived bacteria (colony count)	Per cent survived bacteria of infected (Approximately)	Bacteria from control tube 90 minutes after end of adsorption (colony count)
A Micrococci	2 5 × 10	1 0 × 10^{9}	115 × 10	50 × 10	20	8 × 10^{5}	1	9 2 × 10^{7}
B Micrococci	2 5 × 10^{8}	9 3 × 10^{7}	85 × 10^{8}	45 × 10^{9}	18	8 × 10^{5}	1	1 3 × 10^{8}
C Micrococci	2 5 × 10^{9}	9 6 × 10^{7}	60 × 10^{9}	35 × 10	14	1 × 10^{6}	1	9 5 × 10^{7}
D Micrococci	2 5 × 10^{8}	1 1 × 10	[illegible] × 10^{4}	4 × 10^{4}	0 0002	1 4 × 10^{8}	100	1 3 × 10^{8}
E My(α)	2 5 × 10^{8}	8 6 × 10^{7}	45 × 10^{8}	25 × 10	10	1 8 × 10^{7}	20	9 4 × 10^{7}
F Mw(α)	2 5 × 10^{8}	1 2 × 10^{8}	45 × 10	26 × 10	10	2 × 10^{6}	2	9 7 × 10^{7}

DNA ase treated phage lysate In experiment D use was made of 0 5 ml of undiluted phage lysate passed through a membrane filter of average pore diameter less than 100 mμ (group 10 *Gottingen*) with consequent reduction of the phage titre from 10^{10} to 10^{5} p f u /ml M o i multiplicity of infection Average number of adsorbed phages per bacterium On plates used for colony *count* the number of colonies per plate did not exceed about 200 In order to avoid re infection of bacteria that survived the primary infection *the tubes* with the diluted adsorption mixture and antiserum were kept at +37 C for 90 minutes after the end of adsorption before plating

TABLE 7

Number of Plaques on Titration of a Phage on Surviving Bacteria after Infection of Micrococci with a Phage in Broth

Number of plaques on surviving bacteria				Number of plaques on micrococci	Number of plaques on control bacteria from 10 colonies	Bacteria obtained from experiment (Table 6)
from colonies	1–14	123–161 mean 144		141	121–154 mean 134	A m.o.i. 20
from colony	15	40 (28%)	19%			
from colonies	1–11	114–130 mean 126		122	102–128 mean 112	B m.o.i. 18
from colony	12	18 (15%)	12%			
from colony	13	19 (16%)	11%			
from colony	14	25 (20%)	16%			
from colony	15	29 (24%)	18%			
from colonies	1–13	107–138 mean 118		134	123–149 mean 131	C m.o.i. 14
from colony	14	35 (26%)	18%			
from colony	15	21 (16%)	20%			
from colonies	1–25	42–75 mean 54		66	42–68 mean 57	D m.o.i. 0.000[illegible]

In each of the experiments A, B and C (Table 6) broths were inoculated with surviving bacteria from 15 colonies and from 10 colonies on control plates. In experiment D broths were inoculated with surviving bacteria from 25 colonies and from 10 control colonies. The broths were incubated at +37 C during aeration to a density of 4–5 × 10^8 bacteria/ml. Three titration plates were prepared from each broth. On preparation of the titration plates with bacteria from the same experiment, phage dilution from the same dilution tube was used. From each broth culture a soft agar plate was also prepared for control of the occurrence of any free phage. The value given for the number of plaques is the mean of the numbers found on three titration plates. The numerals given in brackets indicate the number of plaques in per cent of the number on micrococci.

The number of plaques on bacteria from respective colonies after three subcultures on solid medium and three passages in antiphage serum in per cent of the number of plaques on micrococci. The number of plaques on micrococci at this titration was 13.

appeared to facilitate the survival of the bacteria in the presence of the phage.

In the experiment where micrococci were infected with *a* phage, all together about 2 × 10^{9} colonies with surviving bacteria and colonies on control plates were inspected. No white colonies were seen either among the colonies with surviving bacteria or among the control colonies.

Surviving bacteria from 200 colonies from each of the experiments A, B and C were inoculated onto soft agar containing 10^{11} phages/ml. None of the inocula gave growth.

Table 7 gives the results of titration a phage on bacteria surviving infection of micrococci with the phage in broth and on bacteria from control plates.

The number of plaques on the bacteria from the colonies on control plates in the respective experiments did not differ significantly from one another or from the number of plaques on the micrococci. Bacteria from all together 40 colonies on control plates were examined. The values noted for the bulk of the bacteria examined from colonies with surviving bacteria were the same as those from the colonies on control plates in the respective experiments but the number of plaques on bacteria from 7 colonies was significantly smaller. The plaques that developed on these bacteria were of the same morphological appearance as those on micrococci and on bacteria from colonies on control plates.

Bacteria on which a smaller number of plaques were obtained were found only in colonies with surviving bacteria after infection of micrococci with phage in excess and independently of the fact whether the phage lysate was treated with DNA ase or not. Altogether 45 colonies with surviving bacteria after infection with phage in excess were examined.

After adding a phage lysate that had been passed through a membrane filter which retained most of the phages but which presumably allowed the passage of free DNA molecules titration on bacteria from 25 colonies revealed no plate with significantly smaller number of plaques.

The bacteria from 15 per cent (7 out of 45) of the colonies with bacteria surviving infection with phage in excess thus showed a changed relation to the phage which means that on an average 0.15 per cent of the infected bacteria had this property.

After subculture of such bacteria and passages in antiphage serum retitration of the phage showed the same number of plaques as before the first subculture (Table 7). The adsorption of the phage to these bacteria at +37 °C for 30 minutes gave the same values as adsorption to the micrococci.

DISCUSSION

A virulent phage whether wild or a mutant of a temperate phage has no ability to establish lysogeny. Following infection of a bacterium with a virulent phage the phage multiplies vegetatively with consequent lysis of the bacterium. Virulent phages usually produce clear plaques since no bacteria can grow in the area of the plaque.

The α phage produced clear plaques and appeared not to be able to establish lysogeny. The phage thus had the character of a virulent phage but the lytic activity seemed to be modified at +37 °C. About 20 per cent of the phages that developed plaques at +30 °C no longer did so at +37 °C. If secondary growth were to occur on infection of micrococci on solid media the plates had to be incubated at +37 °C. No secondary growth occurred on incubation at +30 °C.

The development of phage resistant mutants of *E. coli* is believed to be a one step event and to imply absolute resistance. The mutation rate

per bacterium per division is of the magnitude of 10^{-6}. Usually phages are not adsorbed to resistant mutants (*Luria & Delbruck* 1943).

Staphylococci not sensitive to phage can however often adsorb the phage but the multiplication of phage is blocked in steps following the adsorption (*Wentworth* 1963). The existence of mutants with relative resistance to phages and due to factors other than lack of phage receptors can therefore not be excluded.

The changed relation to the α phage manifest by a decrease in plaque number on titration and proved to be advantageous for growth of the bacteria in the presence of the phage was not due to a change in the rate of adsorption of the phage but probably to a change in factors affecting the multiplication of phage after adsorption.

On infection on solid media all the secondary growth bacteria examined showed a changed relation to the phage. This property was not stable but changed in the first subculture and could be completely lost. This indicates that the property was due to genetic material introduced into the bacteria and lost during the following generations before it was firmly established in the bacteria.

This is reminiscent of the segregation in phage sensitive bacteria which can occur in several generations after infection with temperate *coli* and *salmonella* phages (*Lieb* 1953, *Levine* 1957, *Uetake et al* 1958).

On infection of a broth suspension about 0.15 per cent of the infected bacteria showed a changed relation to the phage. In these bacteria a stable intergration of the determinants appeared to occur before the first subculture.

Following infection on solid media the bacteria showed changes in their relation to the phage that were more striking than those following infection in broth. This suggests that the degree of the property was related to repeated infection by the phage.

As bacteria with changed relation to the phage were obtained after infection in broth with DNAase treated phage lysate it is probable that the determinants of the property did not occur as free DNA in the lysate but as parts of the genome of the α phage.

Bacteria capable of producing white pigment were obtained only after infection of micrococci on solid media. No segregation of pigment production was noticed in subcultures of these bacteria on solid media or after passages in antiphage serum. The changed relation to the phage in these bacteria was lost during the first subculture.

As the ability to produce white pigment appeared in bacteria without demonstrable association with any property that might favour their growth more than that of other bacteria of the secondary growth it seems reasonable to presume that they were not selected mutants.

It might however be possible that the treatment in antiphage serum for 60 minutes at +37 C in some way induced the development of bacteria capable of producing white pigment but this did not seem to

be the case About 2 × 10⁴ colonies obtained from control bacteria and from surviving bacteria after infection in broth kept for 90 minutes at +37 C in antiphage serum before plating showed no white colonies

In the bacteria capable of producing white pigment no stable determinants affecting the relation to the phage occurred and no bacteria were found in which this determinant was firmly established and which produced white pigment This may suggest a close relationship between two determinants manifest as a dependence of the same region on the bacterial chromosome necessary for proliferation synchronously with the host bacteria like that which may exist between homologous temperate phages (*Bertani* 1953) and indicates that the genetic material determining the production of white pigment also was part of the genome of the *α* phage

However another possibility is that a determinant of the bacterial genome was split off as a result of the phage infection with consequent change in pigment production and loss of the ability of the bacteria to permit any stable intergration of phage genome

Whether infection of micrococci occurred on solid substrates or in broth any bacteria that could be characterized as lysogenic with normal or defect prophages were not found

Any extrabacterial occurrence of phages with re infection of bacteria could not either be demonstrated to be necessary for the bacteria to retain their new properties This contrasts with the situation in so called pseudolysogenic clones infected with converting phages where passage in antiphage serum results in elimination of phages and thereby the property determined by the phage (*Li et al* 1961 *Jones et al* 1962)

On lysogenization of *E coli* with the λ phage two processes are involved which are determined by different regions of the phage genome synthesis of a substance which represses vegetative multiplication and anchorage to the chromosome of the bacterium Anchorage seems to be necessary for growth of the prophage in synchrony with the bacterium (*Zichichi & Kellenberger* 1963) Processes of the same nature seem to be involved on lysogenization of *Salmonella* typhimurium with phage P 22 (*Zinder* 1958)

The results of this study appear to suggest that the genome of the *α* phage contained determinants which to a certain extent could repress vegetative phage multiplication and that these determinants could be firmly intergrated in the bacteria

SUMMARY

Following infection of a micrococcal strain with a newly isolated phage no lysogenized or phage resistant bacteria were found The surviving bacteria could either show a changed relation to the phage manifest by a decrease in plaque number on titration or ability to produce white pigment distinctly different from the yellow pigment produced by un infected micrococci

The results suggest that the genome of the phage contained deter minants which to a certain extent could repress vegetative phage multiplication and that these determinants were firmly intergrated in the bacteria resulting in the changed relation to the phage

The genome of the phage possibly also contained determinants af fecting pigment production with ability of establishing themselves firmly in the bacteria

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INHIBITION BY ACETAZOLAMIDE OF THE GROWTH OF *NEISSERIAE* AT INCREASING ENVIRONMENTAL CONCENTRATION OF CO_2

By

A FORKMAN

Received 7 xii 67

In a previous investigation (*Forkman & Laurell* 1965) it was shown that acetazolamide a potent carbonic anhydrase inhibitor (*Miller et al* 1950 *Woodford et al* 1961) inhibits the growth of *N meningitidis* *N gonorrhoeae* and *N flava* but not that of other bacteria studied The inhibiting effect of acetazolamide on *N meningitidis* and *N flava* was found to be dependent on the contration of CO_2 in the culture milieu when the concentration of CO_2 was high there was little or no inhibition of growth and *vice versa* (*Forkman & Laurell* 1966)

Sanders & Maren (1967) tested ten carbonic anhydrase inhibitors sulphonamides and found that nine of these including acetazolamide inhibited the growth of most Neisseria strains studied This inhibiting effect was noted only when the CO_2 concentration in the culture milieu was low Like *Veitch & Blankenship* (1963) they found that certain pharyngeal *Neisseria* strains produced carbonic anhydrase Only growth of strains that produced carbonic anhydrase was inhibited by carbonic anhydrase inhibitors It was assumed that carbonic anhydrase inhibitors inhibit growth by reducing the formation of bicarbonate

This paper reports more detailed studies of the relation between the inhibiting effect of acetazolamides and the CO_2 concentration in the culture milieu The investigations were carried out on *Neisseriae* capable of growing in air and included some meningococcal and gonococcal strains

MATERIAL AND METHODS

N meningitidis Strains were isolated from the cerebrospinal fluid of patients with meningitis or from the throat of carriers of meningococci Three of the strains had been kept freeze dried

N gonorrhoeae Strains isolated from patients with gonorrhoea

N flava *N perflava* *N subflava* *N catarrhalis* Recently isolated from throat swabs

This investigation was supported by grant from the *Medical Faculty of Lund University*

All of the bacterial strains were differentiated according to *Bergey's* manual (1957) and conventional diagnostic methods

The culture medium Placenta digest agar with horse serum and heated horse blood as described in a previous paper (*Forkman & Laurell* 1965)

The acetazolamide preparation used was Diamox (Lederle) dissolved in sterile 1/15 M phosphate buffer pH 7 4

Determination of the antibacterial effect of acetazolamide A dilution method with solid medium was used (*Reyn et al* 1963 *Forkman & Laurell* 1965) The method was modified in one respect before being streaked on the plates the bacteria were suspended in trypticase broth (Difco) with 25 per cent ascitic fluid instead of placenta infusion broth

Each examination was repeated The results varied within two dilution steps which were the limits of error of the method

Ambient gas milieu The culture was done in an 10 litre air tight box The gas mixture saturated with moisture and warmed was led in at the bottom of the box and out at the top A shallow basin containing water was placed on the bottom of the box The box was placed in a fairly large incubator at 37 C. The minute volume of the ambient air (via an membrane pump) and CO (from a container with 100 per cent CO_2) were regulated by flowmeters The CO_2 flow was varied The air flow was kept constant at 425 ml/min If the airflow exceeded 425 ml/min the air was not heated to 37 C Since the minimum measurable volume of CO by the flowmeter was 2 ml/min the concentration of CO in the gas mixture could not be lower than 0 5 per cent The examination was also carried out in air corresponding to an ambient CO concentration of about 0 03 per cent

RESULTS

Variation of Ambient CO_2 Concentration

In the case of strains of *N meningitidis N gonorrhoeae, N flava N perflava N subflava* and *N catarrhalis* the minimum inhibitory concentrations (MIC) of acetazolamide were determined with the plate dilution method These *N gonorrhoeae* strains could grow satisfactorily in air without supplementary CO The examinations were carried out with CO concentrations of 10 2 and 0 5 per cent in the ambient gas Determinations were also made with air without supplementary CO flowing through the culture box *i e* a CO_2 concentration of about 0 03 per cent (Table 1) Bacterial growth was satisfactory throughout and was not appreciably influenced by a decrease of the CO concentration

The growth inhibiting affect of acetazolamide increased when the CO_2 concentration in the ambient gas was reduced The largest increase of the effect of acetazolamide occurred when the CO concentration was reduced from 0 5 per cent to 0 03 per cent As regards some of the strains the MIC of acetazolamide remained unchanged when the CO concentration was reduced to 0 5 per cent and did not clearly decrease until the CO concentration was reduced to 0 03 per cent

A comparative investigation in 10 per cent CO was carried out on four gonococcal strains capable of growing in air and 10 gonococcal strains that could only grow with an extra supply of CO The MIC of acetazolamide for the 10 gonococcal strains whose growth required supplementary CO_2 ranged between 28 μg/ml and 57 μg/ml As to strains that could grow in air the MIC of acetazolamide ranged from 57 μg/ml to > 912 μg/ml

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INHIBITION BY ACETAZOLAMIDE OF THE GROWTH OF *NEISSERIAE* AT INCREASING ENVIRONMENTAL CONCENTRATION OF CO_2

By

A. Forkman

Received 7.xii.67

In a previous investigation (*Forkman & Laurell* 1965) it was shown that acetazolamide, a potent carbonic anhydrase inhibitor (*Miller et al.* 1950, *Woodford et al.* 1961) inhibits the growth of *N. meningitidis*, *N. gonorrhoeae* and *N. flava*, but not that of other bacteria studied. The inhibiting effect of acetazolamide on *N. meningitidis* and *N. flava* was found to be dependent on the contration of CO_2 in the culture milieu: when the concentration of CO_2 was high, there was little or no inhibition of growth and *vice versa* (*Forkman & Laurell* 1966).

Sanders & Maren (1967) tested ten carbonic anhydrase inhibitors (sulphonamides) and found that nine of these, including acetazolamide, inhibited the growth of most Neisseria strains studied. This inhibiting effect was noted only when the CO_2 concentration in the culture milieu was low. Like *Veitch & Blankenship* (1963) they found that certain pharyngeal *Neisseria* strains produced carbonic anhydrase. Only growth of strains that produced carbonic anhydrase was inhibited by carbonic anhydrase inhibitors. It was assumed that carbonic anhydrase inhibitors inhibit growth by reducing the formation of bicarbonate.

This paper reports more detailed studies of the relation between the inhibiting effect of acetazolamides and the CO_2 concentration in the culture milieu. The investigations were carried out on *Neisseriae* capable of growing in air and included some meningococcal and gonococcal strains.

MATERIAL AND METHODS

N. meningitidis: Strains were isolated from the cerebrospinal fluid of patients with meningitis or from the throat of carriers of meningococci. Three of the strains had been kept freeze-dried.

N. gonorrhoeae: Strains isolated from patients with gonorrhoea.

N. flava, *N. perflava*, *N. subflava*, *N. catarrhalis*: Recently isolated from throat swabs.

This investigation was supported by grant from the *Medical Faculty of Lund University*.

amined 4 *N sicca* strains 3 of which were found to contain carbonic anhydrase These authors could not however demonstrate carbonic anhydrase in *N flava N perflava N subflava* or *N catarrhalis* nor in *Streptococcus salivarius* and *Lactobacillus acidophilus* *Sanders & Maren* (1967) demonstrated carbonic anhydrase in strains of *N sicca, N perflava* and *N mucosa*

The effect of acetazolamide on *N gonorrhoeae* at low ambient CO concentration could be studied only in four strains capable of growing satisfactorily in air without supplementary CO This ability sometimes developed in gonococcal strains that had been repeatedly subcultured On culture in 10 per cent CO the MIC of acetazolamide was higher in the case of such gonococcal strains than in the case of strains requiring supplementary CO_2 In this respect gonococcal strains with the ability to grow in air resembled other types of *Neisseria* not affected by acetazolamide when cultured in a milieu with 10 per cent CO

As regards gonococci cultured in 0 03 per cent CO_2 (air) the MIC of acetazolamide varied widely from strain to strain This may indicate that different strains produce different amounts of carbonic anhydrase A slight qualitative variation in the amount of placenta digest and/or of horse serum in the substrate may also have influenced the results That gonococcal strains capable of growing in air are inhibited by acetazolamide less than other gonococcal strains may perhaps be due to the fact that the former produce more carbonic anhydrase Another possibility is that they can manage their metabolism without the help of carbonic anhydrase for CO fixation

Most of the meningococcal strains studied and the non pathogenic *Neisseria* strains were not inhibited by acetazolamide until the CO_2 concentration had been reduced to 0 5 per cent or less At higher CO concentrations the partial pressure of the CO in the surroundings of the bacteria is apparently high enough for the bacteria to utilize the ambient CO without the aid of carbonic anhydrase This would mean that carbonic anhydrase serves as a permease at low CO pressure

This significance of carbonic anhydrase in the diffusion of CO through biological membranes has recently been studied and discussed by *Enns* (1967) His findings suggest that carbonic anhydrase may be confined to the surface of erythrocytes and other cellular membranes and there facilitate the transport of CO through the membrane by increasing the conversion of CO to bicarbonate ions Such a theory might hold also for bacterial carbonic anhydrase facilitating the transport of the CO through the cell wall *i e* serving as a permease

The *Neisseriae* studied included 4 strains of *N catarrhalis* In all of these cases the MIC of acetazolamide was 456 μg/ml when cultured in air Three of the strains showed a tendency to be inhibited to a higher degree by acetazolamide if they were grown in 10 per cent CO than if they were grown in air without supplementary CO Thus these three *N catarrhalis* strains appear to be more dependent on carbonic anhy

drase if they are to grow at high CO_2 concentration than if they are to grow at low CO concentration *i e* the opposite of features found in cases of other types of *Neisseria* This may suggest that the CO metabolism of *N catarrhalis* differs from that of other types of *Neisseria* studied

SUMMARY

The relation between the growth inhibiting effect of acetazolamide and the CO concentration of the culture milieu was studied using strains of *N meningitidis*, *N gonorrhoeae* *N flava* *N perflava*, *N subflava* and *N catarrhalis* The findings showed that the lowest inhibiting concentration of acetazolamide decreases with the concentration of environmental CO in all of the studied *Neisseriae* except *N catarrhalis*

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BRIEF REPORT

A ROUTINE COUNTERSTAIN FOR THE PAS REACTION

By *Michael Petri*

While a counterstain is often undesirable in analytical histochemical work with the PAS reaction it is of obvious advantage in diagnostic histopathology in which a clear definition of tissue elements is quite essential With delicate PAS positive structures such as the epithelial and glomerular basement membranes of the kidney a good colour contrast is especially valuable The glomerular membrane is particularly important in kidney lesions

During a study of PAS stained formalin fixed kidney tissue counterstained by alum haematoxylin the basal membranes were found to have somewhat hazy outlines due to the reddish shade of haematoxylin which lessens colour contrast considerably A clearer blue non reddish shade was obtained by using *Celestine Blue* which is recommended by *Pearse* (1960) It seemed however that green would be preferable as the complementary colour A 1 per cent aqueous solution of *Fast Green* was tried but it was found to give a rather diffuse tissue stain which was in this respect much inferior to Celestine blue

By using Celestine Blue as a nuclear stain and a saturated aqueous solution of picric acid as a plasma stain a green shade was obtained which combined high contrast with excellent general tissue staining

Celestine Blue was prepared according to *Lendrum* (1951) or *Gray et al* (1956) and both were found satisfactory No qualitative or quantitative difference of the PAS stain was observed between counterstained and non counterstained sections The cold Schiff procedure and periodic acid Schiff stain as prescribed by *Lillie* (1965) were employed

Recommended Procedure

After periodic acid Schiff staining including sulphite rinses the slide is rinsed in water for 10 minutes

Celestine Blue for 2 minutes
Rinse in water for 3 minutes
Saturated aqueous *picric acid* for 2 minutes
Dehydrate in absolute ethanol 3 baths
Clear mount in DPX

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BRIEF REPORT

THE APPEARANCE OF ESTERASES IN HEALING FRACTURES

By *J. Raekallio* & *P. E. Mäkinen*

The activity of several enzymes including esterases increases during the very first post operative hours in healing skin wounds (*Raekallio* 1960, 1965). With regard to fractures there still exists the prevalent view that an inert latent period occurs up to the 2nd or 3rd day after injury (*Bourne* 1948). This seems to be due to lack of investigations into the earliest phase of healing. To study the appearance of esterases in the initial phase of fracture healing we made an experimental investigation demonstrating the enzymes histochemically.

The right tibia of each (4 month old) rat was fractured in the mid diaphyseal region by digital pressure. Groups of 3 rats were killed 1, 2, 4, 8, 10, 12 and 16 hours and 1, 2 and 3 days after fracturing. The tibias were demineralized (*Balogh* 1962). Frozen sections were post fixed and incubated for 10 minutes in a substrate mixture containing α-naphthyl acetate and Fast blue B as the coupler (*Pearse* 1960). Alternate sections were incubated for 40 minutes with Naphthol AS acetate as the substrate and Fast blue BB as the coupler (*Pearse* 1960).

At 1 to 8 hours after the injury the fracture defect was filled and surrounded by extravasated blood and inflammatory exudate. Numerous polymorphonuclear leucocytes with esterase positive cytoplasm appeared in the exudate after 4 hours. At 10 hours and later on the first signs of increase in esterase activity were noticed in the osteoblasts and undifferentiated osteogenic cells of the periosteum. Esterase activity of these cells and of osteoclasts demonstrable by both of the methods firstly elevated in a peripheral zone situated at a distance of 200–500 μ and farther away from the fracture line. At 16 hours and subsequently an initial proliferative response was seen in the periosteum. This occurred in the same peripheral zone which had showed the first increase in esterase activity at 10 hours. The proliferation became more evident at 24 hours and during the 2nd and 3rd day. The proliferating periosteal cells showed an intense esterase activity. Similar phenomena were observed in the endosteum 3 days after the injury the initial blood clot around the fracture ends was replaced by granulation tissue showing intense esterase activity.

The first reaction to fracture is essentially a sterile inflammation which is the consequence of trauma and haemorrhage. Also in the local periosteal cells there is a very early enzymatic response to injury. Trauma affecting the multipotent periosteal cells seems to serve as a stimulus activating defence forces after a relatively short mobilization time. Besides functioning as a defence barrier the enzymes may play a part in more specific regenerative processes since esterases have been correlated (*Savchuck & Burstone* 1958) with an early stage of calcification.

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GROWTH IN RAT OF A SARCOMA DERIVED FROM RAT KIDNEY CELLS TRANSFORMED IN VITRO BY SV40

By

REBEKKA BERG and UNNE STENRAM

Received 5 xii 67

In a previous communication transformation of rat kidney cells *in vitro* by SV40 virus and the properties of the transformed cells were described Tumours were obtained by autologous transplantation in 4 X irradiated recipients The tumour from one rat was examined histologically and characterized as a spindle cell fibrosarcoma with a moderate cell polymorphism and abundant mitoses It was found to contain the complement fixing (CF) SV40 neoantigen (*Diderholm et al* 1966)

A tumour from another rat was used for transplantation Some growth characteristics of the rat sarcoma line obtained in this way and the results of macroscopical and histopathological examinations of tumour bearing rats from the first 14 passages are described in the present report

MATERIALS AND METHODS

Tumour passages Transplantation of the tumour was performed in 3 week old male rats of the Sprague Dawley strain (*Diderholm et al* 1966) The first tumour was transmitted to 10 rats 6 of which obtained 400 r total body X irradiation The following passages were routinely performed in non irradiated rats using about 50×10^6 cells of trypsinized tumour tissue The cells were resuspended in 2 ml Eagle s minimal essential medium (MEM) with antibiotics and injected subcutaneously and intramuscularly into the back above the tail

In the 13th and 14th passages the tumour growth was studied after inoculation via four different routes Each rat in the first group consisting of 7 animals obtained 30×10 cells subcutaneously and in the second group (6 animals) the same amount of cells was given intraperitoneally In the third group (8 animals) each animal obtained 3×10^6 cells into the subdural space by piercing the parietal bone with the injection needle and in the fourth group (4 animals) the same amount was injected intravenously into a tail vein near the root of the tail

Tumours from the first 14 passages in a total of about 400 animals constitute the material studied and described in the present publication

Morphological technique At autopsy the tumour and the thoracic and abdominal organs were inspected and as a rule also the brain Pieces of tumours lymph nodes brain and visceral organs were taken for histological examination They were fixed

This work was supported by grants from the *Swedish Cancer Society* (Projects C7 31 and 67 57) and the *Swedish Medical Research Council* (Project K67 12X 6 3 03)

in 10 per cent f rmalin and stained with haematoxylin-eosin and haematcxylin van Giesen.

Tumours from later passages were also fixed in OsO and prepared for studies in a Siemens electron micrcs ope (Elmiskop 1)

Trypsinized tum ur cells were cultivated in Leighton tubes fixed in acetone and stained with a fluores ent serum from SV 0 tumour bearing hamsters (Flow Laboratories Ltd., Irvine Scotland) according to the technique described by *Rapp et al* (1964)

Assay of CF a tivity A 20 per cent (v/v) suspension in MEM with antibi tics of hom genized fresh tum ur tissue was used as an antigen in CF tests with serum from SV40 tum ur bearing hamsters as described before (*Diderholm et al* 19CC) CF tests were als perf rmed with an SV40 neoantigen from transformed bovine tissue (*Diderholm et al* 19C5 *Diderholm & Wesslen* 19C5) and sera from tumour bearing rats These sera were taken by heart puncture before tumour transplantation and immediately before the animals were sacrificed

Assay f infecti ns SV40 The same suspensi n was tested for the presence of SV40 as described previ usly (*Dideth lm et al* 19 C)

Assay of tum ur producing capacity Trypsinized tumour cells were diluted in MEM with antibi tics and inoculated subcutanec usly in male 3 week-old Sprague Dawley rats. Cell viability of the undiluted suspensi n was tested by adding trypan blue to a final concentration of 0.2. per cent.

OBSERVATIONS

Transplantability of the Tumour

Transplantation of cells from the first tumour gave progressively growing tumours in all 6 irradiated and in 2 out of 4 nonirradiated animals These tumours were all of the same appearance as the first tumour obtained and grew well delimited without infiltration and metastases From the fourth passage all animals obtained progressively growing tumours usually killing them within a month (Table 1)

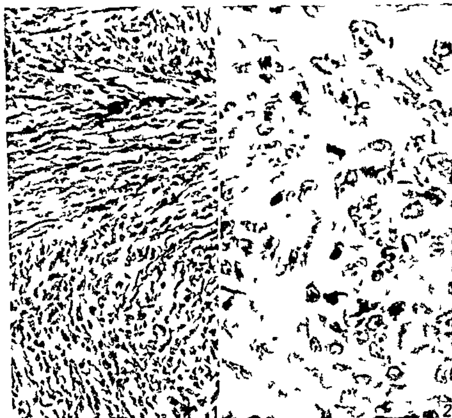
TABLE 1

The Frequencies of Tumours and Metastases in Non Irradiated Animals Table Gives Number of Animals with Tumours over Total Number of Animals

Passage number	Subcutaneous tumour	Metastases to regi nal lymph nodes	Pulmonary metastases
2	2 4	0 4	0 4
3	6 9	2 9	0/9
4	10 10	1 10	0/10
5	10 10	9 10	3/10
13	7 7	6 7	6 7

Growth Appearance of the Tumour

Two to five days after disappearance of the post inoculation soft oedematous swelling small hard nodules appeared which gradually coalesced to form large firm tumours greyish white on the cut surface Different degrees of ulceration and central liquefaction necroses were common in slowly growing tumours



Figs 1 2

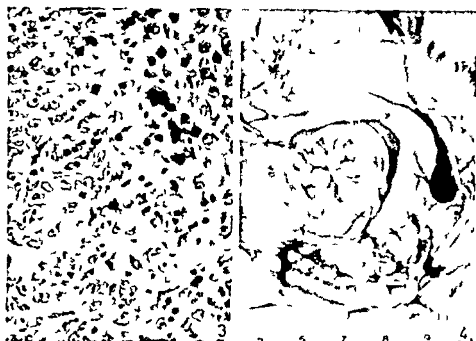
Fig 1 Primary tumour showing growth of tumour cells in fascicles (Passage 1) Haematoxylin van Gieson × 250

Fig 2 Primary tumour Nuclear details and a mitotic figure are seen (Passage 2) Haematoxylin van Gieson × 630

The well delimited subcutaneous growth of the tumour in the first two passages changed into infiltrative growth in later passages with invasion into the muscles spine and internal organs

In the 13th passage rats were inoculated with 30 × 10 cells either subcutaneously or intraperitoneally After subcutaneous administration rats died after 14 to 21 days After intraperitoneal inoculation the rats were sacrificed when moribund after 5 to 14 days At autopsy the bellies of the latter animals were grossly distended containing about 100 ml of serosanguinolent ascites with lots of small clumps of tumour cells *Carcinosis peritonei* was seen in all animals In 4 out of 6 animals the tumour grew in radial strands in the mesenterium and along its attachment to the small intestine (Fig 4)

After subdural administration of cells the animals died within 11 to 19 days In 5 out of 8 rats tumours grew extracranially at the site of



Figs 3 4

Fig 3 Tumour gro th in the liver N te the large nuclei and nucleoli compared t Figs 1 and 2 Normal liver cells are seen in the upper right corner (Passage 6) Haematoxylin-eo in × 2?0

Fig 4 Intraperitoneal inoculation Tumour growth on the parietal peritoneum and like the spokes of a wheel in the mesenterium (Passage 13)

the injection All rats had tumours growing as a shell over the convexity of the brain

After intravenous administration of tumour cells the animals were sacrificed when moribund after 16 to 20 days Tumours were found subcutaneously at the root of the tail in the pelvic region and often in the lungs (see under Metastatic spread)

Optical Microscopy

The tumours (Fig 1) were cell rich spindle-cell sarcomas with sparse connective tissue fibrils and with numerous round or spindle shaped cells arranged in an irregular network of bundles The nuclei were round or elongated and varied in stainability As a rule they were vesicular with one or sometimes several distinct nucleoli but were sometimes fairly rich in chromatin (Fig 2) Usually the cytoplasm was scanty and the mitoses were abundant Occasional multinucleated giant cells were found Several small scattered necrotic areas were sometimes found in the primary tumour The inflammatory reaction in the periphery of the tumours was slight with few lymphocytes

The sarcomatous appearance of the tumour was retained in all pas



Fig 5

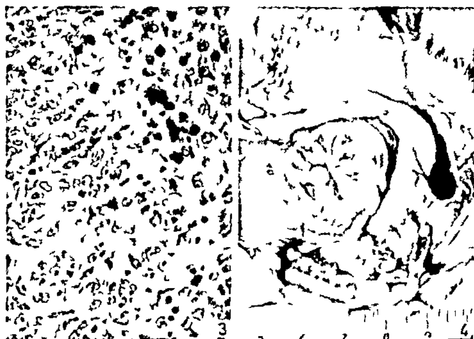
Intracranial inoculation The tumour grows on the surface of and perivascularly down into the brain (Passage 13) Haematoxylin van Gieson × 160

sages but the tumour cells nuclei and nuclei appeared to be larger (Fig 3) and the mitotic figures more numerous in later passages

Tumours obtained after intraperitoneal administration showed the same histological picture as the other tumours After subdural administration of cells tumour grew in all rats as a shell over the brain and extended down into the brain along the vessels (Fig 5) No ependymomas were found

Metastatic Spread

Metastases were sporadically found from the 3rd passage in the lumbar nodes and from the 5th passage regularly in the draining lymph nodes and occasionally in the lungs (Table 1) After intraperitoneal administration metastases were found in lymph nodes along the aorta and in the lungs in about 50 per cent of the rats No metastases were found after subdural administration of tumour cells Metastases to lymph nodes were found in 2 out of 4 rats intravenously injected and to the lungs in 3 out of 4 animals The metastases contained less connective tissue fibrils than the primary tumour and were less necrotic but were otherwise of the same histological appearance (Fig 3)



Figs 3 4

Fig 3 Tumour growth in the liver Note the large nuclei and nucleoli compared to Figs 1 and 2 Normal liver cells are seen in the upper right corner (Passage 6) Haematoxylin eosin × 275

Fig 4 Intraperitoneal inoculation Tumour growth on the parietal peritoneum and like the spokes of a wheel in the mesenterium (Passage 13)

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Figs 6–7

Electron micrographs of primary tumour OsO_4 fixation (Passage 13) Uranyl acetate lead citrate staining

Fig 6 General survey showing parts of three cells The nucleus in the lower left corner contains a nucleolus with a well developed nucleolonema In the cytoplasm are seen a few mitochondria scantines of endoplasmic reticulum and lots of free ribosomes and polysomes × 17 500

Fig 7 Microvilli are seen on the surface of cells In the cytoplasm there are free ribosomes and polysomes × 43 000

Ultrastructure

The cell nuclei were only slightly indented The nucleoli had well recognizable nucleolonemata but varied in size and shape There was a rather small amount of lysosomes mitochondria and endoplasmic reticulum but most cells were rich in polyribosomes or free ribosomes (Fig 6) The cell surface was often rich in microvilli (Fig 7) Virus like particles were not observed

TABLE 2

CF Antigen in Rat Tumours Demonstrated with Serum from SV40 Tumour Bearing Hamster and CF Antibody Titre from the Serum of the Tumour Bearing Rat Tested against SV40 Transformed Bovine Tissue

Passage number	Antigen titre of tumour	Antigen titre of serum
1	1/4	1/32
2	1/2	1/32
3	1/2	1/128
4	1/4	1/32
5	1/4	1/32
6	1/4	1/128

Presence of SV40 Neoantigens in the Tumours

The immunofluorescent tests on tumour tissue with sera from tumour bearing hamsters consistently gave positive results. The characteristic picture (*Rapp et al.* 1964) with fluorescent nuclei was found in all passages tested.

Titrations of suspensions from the tumours showed that an antigen reactive with serum from SV40 tumour bearing hamsters was present in all tumours examined and that the titres were unaltered throughout the passages. Table 2 shows the results from single animals from passages 1 to 6. As also demonstrated in the table, all these animals had responded with fairly high titres of antibodies against a transformed bovine tissue containing SV40 neoantigen. In all 47 tumour bearing rats were tested for the presence of serum antibodies. The results are given in Fig. 8.

Lack of Infectious SV40 Virus

Selected tumours in each passage from passages 1 to 11 were tested for the presence of infectious SV40. In no instance could infectious virus be demonstrated.

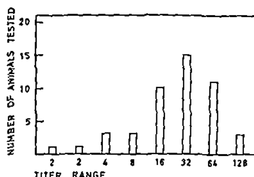


Fig. 8

Antibody titres in serum from tumour bearing rats tested against SV40 transformed bovine tissue

TABLE 3

Number of Viable Tumour Cells at Different Passage Levels giving Tumours in 3 Week Old Rats Table Gives Number of Rats with Tumours over Total Number of Rats

Passage number	Number of viable cells injected					
	5×10^6	10^6	10^5	10^4	10^3	10
3	3/3	0/3	ND	0/3	ND	0/3
10	6/6	6/6	2/6	3/6	2/6	0/6
11	6/6	6/6	5/6	2/6	1/6	0/6
11	5/6	0/6	0/6	ND	ND	ND

Tests performed in 8 week old rats   ND = not done

Tumour Producing Capacity

Table 3 shows that the 50 per cent tumour producing cell dose decreased by a factor of about 100 between passage 3 and passage 10-11 8 week old rats required about 100 times more cells than 3 week old animals to develop the same tumour response when cells of the 11th passage were used Some earlier tumour passages performed in older animals had given negative results in several animals

DISCUSSION

The tumour described in the present paper was initiated by autologous transplantation of rat kidney cells transformed *in vitro* by SV40 The rat is resistant to the oncogenic action of SV40 *in vivo* It is therefore of special interest that a malignant tumour can be obtained by SV40 also in this species by using another method than virus inoculation of newborn animals SV40 neoantigens were consistently found in all nuclei and all tumour tissue tested in CF test against a serum from SV40 tumour bearing hamsters contained the CF antigen The histological picture of the tumour was uniform and that of a spindle cell fibrosarcoma No carcinomatous elements were found The sarcoma showed a more infiltrative growth and increasing metastasizing capacity with increased number of passages *in vivo* The histological picture of the tumours was also more malignant in later passages

Homologous transplantation of hamster embryo cells transformed *in vitro* by SV40 has been performed by *Ashkenazi & Melnick* (1963) and by *Diamandopoulos & Enders* (1966) the former authors obtaining highly pleomorphic sarcomas the latter carcinomas and sarcomas In experiments with transformed kidney tissue of newborn and embryonic hamsters the tumours obtained contained both sarcomatous and carcinomatous elements (*Ralston & Kirschstein* 1962 *Black et al* 1966) The usual way of producing SV40 tumours i e inoculation of the virus into newborn animals has resulted in sarcomas in hamsters very similar to those reported here though apparently with a larger

amount of multinucleated cells (*Eddy et al* 1961 *Girardi et al* 1962) Ependymomas have been produced by both subcutaneous (*Rabson et al* 1962) and intracerebral (*Gerber & Kirschstein* 1962) inoculation of the virus No such tumours were obtained by us *Kimoto & Croce* (1965) obtained fibrosarcoma haemangiosarcoma as well as intestinal carcinoma by inoculation of SV40 DNA into newborn hamsters Compared with the SV40 induced tumours encountered in literature the tumour described here seems to have an unusually uniform histological picture

Ultrastructural studies are few The ependymomas reported by *Rabson et al* (1962) showed abundance of microvilli and absence of virus particles and were in these regards similar to our tumours

Using the same method as in the present investigation *Dalerholm et al* (1965) obtained a sarcoma in calf after autologous transplantation of SV40 transformed subcutaneous tissue and *Wesslén et al* (1967) and *Wesslén* (to be published) have described the development of a sarcoma in mouse after isologous transplantation of SV40 transformed mouse embryo tissue With a very similar technique *Allstein et al* (1967) failed to obtain tumours in rats after allogeneic transplantation of SV40 transformed embryonic cells Tumours which later regressed however were obtained when the transformed rat cells were inoculated into the cheek pouch of hamsters Human cells can also be transformed by SV40 *in vitro* (*Shein & Enders* 1962 *Jensen et al* 1964) After homologous and autologous implantation of the transformed cells the latter authors found nodules which regressed They had a sarcomatous pattern of growth Autologous implantation of monkey cells transformed *in vitro* by SV40 failed to produce tumours (*Rabson et al* 1965)

Experiments with transplantations of SV40 transformed tissue have given both sarcomatous and carcinomatous tumours in hamsters which are also sensitive to the oncogenic action of SV40 *in vivo* Transplantation in different virus resistant animals however have so far given only sarcomatous tumours but only a few experiments have been performed hitherto Further investigations will show whether it is possible to obtain a tumour from any type of tissue by transformation *in vitro* by SV40 and serial transplantations in irradiated and non irradiated animals as described in the present paper for the rat sarcoma originating from *in vitro* transformed kidney tissue

SUMMARY

The morphology of a transplantable sarcoma derived from a rat kidney transformed by SV40 *in vitro* is described During continuous homologous passage *in vivo* the number of cells required to produce tumour decreased and the tendency to metastasize increased

SV40 neoantigen was demonstrated by immunofluorescence in all

tumour cells in different passages Sera from tumour bearing rats contained CF antibodies against SV40 transformed bovine cells No infectious SV40 was detected

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GROWTH KINETICS OF THE MOUSE EPIDERMIS AFTER A SINGLE APPLICATION OF CIGARETTE SMOKE CONDENSATES

By

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Received 17.x.67

Tobacco smoke condensates induce hyperplasia of the epidermis. The sebaceous glands are affected also and partly disappear. These phenomena are the basis for the combined sebaceous gland and hyperplasia test developed and routinely used at our institute as a short term screening test for carcinogenicity of tobacco tars and other kinds of hyperplasia inducing compounds (*Lazar et al.* 1963). This test has been elaborated empirically. It would therefore be of interest to examine the kinetics of the epidermal cell population after application of various tobacco smoke condensates. Differences in growth patterns might thus possibly be related to the results found by the sebaceous gland and hyperplasia test.

The aim of the present study was accordingly to follow some growth parameters of the epidermal cell population after topical application of different tobacco smoke condensates which first had been tested for carcinogenicity by the sebaceous gland and hyperplasia test. Three growth parameters were followed: the mitotic count, the mitotic rate, and the mitotic duration (for precise definitions of these terms see *Elgjo* 1967).

MATERIALS AND METHOD

Hairless mice (hr/hr) were used in all experiments. The normal epidermal cell population kinetics and its response to topical application of hyperplasia inducing agents are well examined in this strain of mice (*Iversen & Evensen* 1962, *Skjæggestad* 1964, *Elgjo* 1966). The normal values of the growth parameters followed in the present study are as follows (*Elgjo* 1966):

Mitotic count (per 6 mm interfollicular epidermis): 6.74 ± 1.29
Mitotic rate (per 6 mm interfollicular epidermis per hour): 6.36 ± 1.19
Mitotic duration (hours): 1.07 ± 0.016

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The work reported in this paper was undertaken during the tenure of an *Eleanor Roosevelt Cancer Fellowship* of the *American Cancer Society* awarded by the *International Union Against Cancer*.

Four samples of cigarette smoke condensates (A B C D) were examined The condensates were kindly furnished by SEITA When tested by the sebaceous gland and hyperplasia test all four condensates acted as weak carcinogens in the order A>B>C>D (i e A was the strongest and D the weakest carcinogen) A 30 per cent solution in acetone was used both in the test and in the present study of the epidermal cell population kinetics

The animals were divided randomly into 4 groups and each group was treated with 0 05 ml of one of the condensates The condensates were applied to the inter scapular area of the back by means of a calibrated syringe Groups of 8 mice were sacrificed 1 2 3 and 7 days after the treatment (for further details about the ex perimental procedure see *Elgjo* 1968)

The Colcemid method was used to estimate the mitotic rate and the mitotic duration These two parameters were evaluated by means of the following equa tions $R = \frac{C}{t}$ and $D = \frac{M}{R}$ where R is the mitotic rate C the number of arrested mitoses 1 hours after the injection of Colcemid D the mitotic duration and M the mitotic count without injection of Colcemid In the experiments t = 4 hr All values refer to estimates per 6 mm interfollicular epidermis (for discussion of the method see *Eigsti & Dustin* 1955 and *Elgjo* 1966)

TABLE 1

Single Application of 0 05 ml of 30 per Cent Acetonic Solutions of Various Tobacco Smoke Condensates

Time after application	Mitotic count without Colcemid	SE of the mean	Mitotic count after Colcemid	SE of the mean	Mitotic rate	Mitotic duration
			Condensate A			
1 day	8 75	0 76	72 75	6 32	18 19	0 48
2 days	13 75	39	68 50	9 44	17 12	0 80
3 days	3 25	0 72	20 75	5 05	5 19	0 63
7 days	7 2	2 06	43 50	5 72	10 87	0 67
			Condensate B			
1 day	9 00	0 70	58 50	4 61	14 02	0 61
2 days	13 50	1 73	44 25	4 55	11 06	1 22
3 days	6 00	1 41	30 00	5 98	7 50	0 80
7 days	8 25	1 38	41 25	8 79	10 31	0 80
			Condensate C			
1 day	5 75	0 86	65 75	5 61	16 44	0 35
2 days	6 00	0 70	53 25	7 43	13 31	0 45
3 days	3 50	1 50	43 50	4 33	10 87	0 33
7 days	9 75	1 65	39 50	7 92	9 87	0 99
			Condensate D			
1 day	6 25	0 50	59 25	10 28	14 81	0 42
2 days	11 75	2 34	51 75	7 50	12 94	0 91
3 days	3 00	0 91	22 50	0 96	5 6	0 54
7 days	11 75	1 93	29 25	4 00	7 31	1 61

RESULTS

The results are reported in Table 1 and Fig 1 One day after the treat ment the mitotic rates were increased to values about 3 times the nor mal after application of any of the condensates The stronger conden

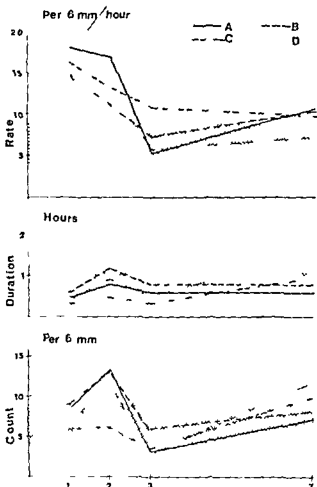


Fig 1

The mitotic rate duration and count the first 7 days after a single application of 0 05 ml of 30 per cent acetonic solutions of four cigarette smoke condensates (A B C and D) of different activity as estimated by the sebaceous gland and hyperplasia test

sate (*A*) provoked however a somewhat higher rise in the rate than did the other samples During the two subsequent days the mitotic rates decreased Apart from the mitotic rate found after application of condensate C all rates were within the normal limits by the 3rd day During the next 4 days the mitotic rates tended to increase and by the 7th day all rates but one were significantly increased Only the rate observed after application of the weakest condensate (D) failed to show this rise

During the initial phase of rapid cell proliferation the mitotic durations were very short mostly about half the normal time The mitotic durations tended to increase relatively however as the mitotic rates

declined After application of condensate B the mitotic duration was even longer than normal on the 2nd day after the treatment During the remaining part of the experimental period the mitotic durations showed different trends After application of condensate A and B the mitotic durations were consistently shorter than normal After treatment with condensate C it was normal by the 7th day and in the experiment with condensate D it even increased to values well above the normal by the end of the experimental period

The mitotic count revealed alterations that partly were due to changes in the mitotic rate partly to alterations in the mitotic duration The changes in the mitotic count thus give inaccurate information about the actual growth pattern as it reflects the alterations of two independent variables

DISCUSSION

The alterations of the growth parameters induced by the various condensates had all characteristics of a simple regenerative reaction the initial increase of the rate of cell proliferation being the response to the cell loss induced by the agents (*Iversen & Evensen* 1962 *Iversen & Bjerknes* 1963 *Skjæggestad* 1964) The inverse relationship between the mitotic rate and the mitotic duration regularly seen in such reactions is also apparent in the present findings

Probably the degree of the initial rise of the mitotic rate is simply related to the amount of cell loss induced by the applied agent (*Skjæggestad* 1964) On the other hand previous studies have indicated that the pattern of the alterations induced by various agents may be to some extent dependent upon the nature of the substances applied (*Elgjo* 1966 *Elgjo* 1968) A single application of a non carcinogenic hyperplasia inducing agent usually provokes a rapid rise in the mitotic rate with a peak value within the first 24 hours During this initial period of rapid cell proliferation the estimated mitotic duration is consistently found to be shorter than normal After a single application of carcinogenic hydrocarbons however the initial period of increased mitotic rate tends to be more sustained and the peak value is usually found between the 2nd and the 4th day Moreover the mitotic duration tends to be longer relatively or absolutely than that found after non carcinogens (for discussion of this see *Elgjo* 1966)

Looked upon from this point of view it is apparent that the alterations of the growth kinetics of the epidermal cell population induced by the cigarette smoke condensates resemble those found after treatment with non carcinogenic agents Table 1 and Fig 1 reveal too that no qualitative differences existed between the growth patterns observed after treatment with the various condensates

Crude cigarette smoke condensates are now recognized as complete although weak carcinogens for a variety of epithelial tissues for re

view see *Wynder & Hoffmann* 1964). And as mentioned above all four condensates used in the present study acted as weak carcinogens when examined by the sebaceous gland and hyperplasia test.

Why then were the alterations of the epidermal cell population kinetics provoked by these condensates similar to those induced by non-carcinogenic hyperplasia-inducing compounds?

One obvious reason could be that the growth pattern previously observed after applications of various carcinogenic agents (*Elgjo* 1968) was due solely to particular properties of the compounds selected for examination and that it does not represent a general principle. Only the examination of more compounds, carcinogenic and non-carcinogenic, can give a definite answer to this problem.

Another reason that might account for the uniform and unspecific growth patterns found in the present study could be that the amount of condensates applied to the skin was too small to elicit the carcinogen-like effect. Several investigations have indicated that carcinogens appear to have some threshold dose when applied topically to the skin (*Poel* 1959, *Terracini et al* 1960, *Iversen & Evensen* 1962, *Iversen & Iversen* 1964). When the amount of applied carcinogen is below the threshold dose of the compound it cannot alone act as a complete carcinogen to the skin. The cigarette smoke condensates contain a number of chemical substances potentially carcinogenic for the mouse skin (see for instance *Roe* 1962, *Wynder & Hoffmann* 1964, *Lazar et al* 1966). The threshold dose of these mixtures of complete and incomplete carcinogenic agents are not known. Another unknown factor is the mode and the extent of penetration into the skin of acetonic solutions of the examined condensates. After a single application a crust of tar remains on the skin surface for about two days. This indicates that only some of the applied amount of condensate has penetrated into the skin. Furthermore *Terracini et al* (1960) have demonstrated that a certain dose of a carcinogen gives a greater tumour yield applied repeatedly in small amounts than when given as a single topical application. The sebaceous gland and hyperplasia test implies 3 successive applications of 0.05 ml of the various compounds. This difference in dosage and way of treatment could therefore possibly explain why the condensates gave positive results when tested by the sebaceous gland and hyperplasia test, while the alterations of the growth kinetics of the epidermal cell population were similar to those usually found after non-carcinogenic agents.

On the other hand the reason why the various condensates provoked almost the same alterations in the growth patterns has recently been suggested (*Chouroulinkov et al* 1967). When a certain amount of such condensates are applied to the mouse skin in increasing concentrations the induced hyperplasia (as estimated by weighing the epidermis) is roughly proportional to the applied dose up to a certain limit. Application of concentrations above this limit gives no further increase of

the hyperplasia The concentration used in the present study exceeds this limit This explains well why the initial rise in the mitotic rate was of the same order of magnitude after application of any of the condensates But it does not indicate why all four condensates failed to provoke a pattern similar to that found after treatment with hydrocarbon carcinogens

Whatever the reason may be for the uniform and unspecific response observed after a single application of cigarette smoke condensates with different activity (as estimated by the sebaceous gland and hyperplasia test) the present study suggests that the following conclusions can be drawn

The difference in activity between the various condensates found by the sebaceous gland and hyperplasia test cannot be directly correlated with differences between the induced alterations in the growth patterns of the epidermal cell population This view is in conformity with the conclusion drawn by *Dontenwill et al* (1966) after experiments with radio autography after labelling with tritiated thymidine The uniform growth pattern induced by the various condensates indicates that the specificity of the sebaceous gland and hyperplasia test possibly relies upon quantitative and not upon qualitative differences between the alterations provoked by the carcinogenic and non carcinogenic compounds respectively Further investigations to clarify this point would certainly be of great interest

SUMMARY

The initial reaction of the epidermal cell population of the hairless mouse (hr/hr) was evaluated after a single application of four cigarette smoke condensates When tested with the sebaceous gland and hyperplasia test all four condensates acted as weak carcinogens The alterations induced in the growth kinetics of the epidermis were examined by following the mitotic rate duration and count The mitotic rate and the mitotic duration were estimated by means of the Colcemid method The results revealed that the alterations provoked in these parameters by the condensates were similar to those observed in simple regenerative reactions The relationship between the induced alterations in the growth pattern of the epidermis and the results obtained with the sebaceous gland and hyperplasia test is discussed

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THE EFFECT OF AN ORAL CONTRACEPTIVE AS A PREPARATORY MECHANISM IN THE GENERALIZED SHWARTZMAN REACTION IN RABBITS

By

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Received 6 xi 67

Investigations of the generalized Shwartzman reaction (GSR) after endotoxin injection in rabbits prepared by means of steroid hormones have been published (*Thomas et al* 1952 *Cooper & McKay* 1960 *McKay & Merriam* 1960) Furthermore clinical studies have suggested a correlation between female sex hormones and some coagulation disorders such as thrombocytopenic purpura (*Pepper et al* 1956) and extensive investigations have demonstrated changes in coagulation and fibrinolysis during treatment with oral contraceptives (*Brakman et al* 1967)

The present paper deals with the changes observed in some of the blood coagulation parameters (factor V PP and PTT) and the presence of microthrombi in the organs of female rabbits subjected to intravenous injection of endotoxin following preparation by an oral contraceptive alone or combined with ACTH or chorionic gonadotrophin as compared to pregnant rabbits normal rabbits and rabbits subjected to the classical Shwartzman procedure

MATERIAL AND METHODS

Forty four hybrid female adult rabbits weighing between 1 5 and 2 5 kg were used They were divided into six groups and treated in the following manner

Group I Eight rabbits treated daily for 15 to 30 days with Enavid®[1] (1 2? mg of norethynodrel + 0 018 mg of mestranol) in aqueous solution given by means of a plastic catheter placed in the pharynx After hormone treatment four of the eight rabbits were intravenously injected with endotoxin in doses of 1 4 to 5 6 mg Two rabbits served as controls and were not injected with endotoxin Two rabbits died spontaneously during the hormone treatment

Our sincere thanks to Dr *Ole K Albrechtsen* for his kind advice and constructive criticism in the elaboration of this manuscript to Drs *F Kissmeyer Nielsen* and *N P R Laursen* for the facilities and technical advice provided by them at the Blood Bank Laboratory Municipal Hospital Århus and to Mrs *D Eil rt n* and *A I Osterby* for their kind technical assistance

[1] Enavid® (Searle Chicago)

Group II Seven rabbits treated for 15 to 30 days with Fnavid® as described above together with Acton prolongatum®[2] (5 I U of ACTH gel) injected every second day in the hind limbs Four out of the seven rabbits were given endotoxin after the hormone treatment in doses as above One rabbit served as a control and was not endotoxin injected Two rabbits died spontaneously during the hormone treatment before receiving any endotoxin

Group III Seven rabbits treated for 15 to 30 days with Fnavid® and with Physex®[3] (100 I U of Chorionic gonadotropin) which was given from the 10th day of Fnavid treatment intramuscularly twice weekly Four out of the seven rabbits were given endotoxin as above One rabbit served as a control and was not endotoxin injected Two rabbits died spontaneously during the hormone treatment before receiving any endotoxin

Group IV Eight rabbits approximately 15 days pregnant Five were injected with endotoxin in doses of 2 1 to 4 9 mg Three rabbits were used as controls and did not receive any endotoxin

Group V Ten rabbits non pregnant non pretreated Eight were injected with endotoxin in doses of 1 4 to 7 0 mg Two served as controls and were not given endotoxin

Group VI Four rabbits were given 1 4 mg of endotoxin as a preparatory dose and 2 8 mg of endotoxin as a provocatory dose 24 hours later

Details concerning the duration of the hormone treatment in Groups I to III and the doses of endotoxin in individual rabbits are shown in Tables 1 and 2

Blood samples were taken from rabbits of Groups I to III at time intervals of several days in order to study modifications of the analysed coagulation parameters during hormone treatment (Table 1) Blood samples were secured (from rabbit in Groups I to V) immediately before the endotoxin injection (or the fictitious time for it in the controls) and 1/2 hour 1 3 6 12 and 24 hours later In Group VI blood samples were collected immediately before the second endotoxin injection and 1 hour 3 6 12 and 24 hours later

The blood samples which were obtained by puncture with siliconized hypodermic needles from an ear artery were collected in chilled polyethylene tubes each containing 0 5 ml of 3 8 per cent sodium citrate to 4 5 ml of blood and kept for no longer than 10 minutes at 4 C before centrifugation The separated platelet poor plasma was kept in polyethylene tubes at —20 C for a period not exceeding 24 hours

Determination of factor V was done according to the quantitative method of *Qu k & Stefanini (1948)* using human plasma deficient in factor V The prothrombin proconvertin (PP) was determined following the technique of *Owren & Aas (1951)* using ox plasma deficient in factor VII The partial thromboplastin time (PTT) was measured according to *Proctor & Rapaport (1961)* using the anticoagulant stat d above Results are given in seconds as the average of two estimations differences between which were not greater than 1 second for factor V 2 seconds for PP and 5 seconds for PTT Whenever differences were greater the tests were repeated

After death or sacrifice of the rabbits autopsies were quickly performed Sections of tissue were fixed in 4 per cent formaldehyde solution embedded in paraffin cut and stained with phosphotungstic acid haematoxylin (PTAH) Lendrum stain for fibrin and haematoxylin eosin On the basis of PTAH stained sections from heart lungs spleen liver and kidneys the presence of fibrin thrombi were semiquantified in the following way 0 no thrombi in the section (about 1 cm to 1 5 cm) 1 one *thrombus 2 more than one but less than ten thrombi 3 more than ten thrombi* 4 thrombi in almost every vessel in the section —When more than one section from an organ was made (*i e* kidneys lungs) an average value was calculated From the values obtained in each of the five organs an average for each rabbit was calculated (individual index) Histological examinations were performed blindly without any information on the experimental procedure or the results of coagulation analyses at the time of examination

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Acton prolongatum® (Brdr Vermehren og Ludvigsen A/S Copenhagen)

[3] Physex® (Løvens Kemiske Fabrik Copenhagen)

These hormone preparations were kindly supplied by the above mentioned firms

[4] Lipopolysaccharide B of Escherichia coli 055 B5 (Difco Laboratories Detroit Mich) dissolved in non pyrogenic physiological saline and kept at 4 C in sterile ampules each containing 14 mg of endotoxin per 1 ml

TABLE 1

Modifications in Factor V Prothrombin Proconvertin (PP) and Partial Thromboplastin Time (PTT) in Rabbits Treated with Enavid® (Group I) Enavid® and ACTH (Group II) and Enavid® and Chorionic Gonadotrophin (Group III)

Group I						Group II						Group III					
Rab bit No	Before hor mone treat ment V PP PTT	After 4 days of treat ment V PP PTT	After 10 days of treat ment V PP PTT	At the end of treat ment V PP PTT	Dura tion of hor mone treat ment	Rab bit No	Before hor mone treat ment V PP PTT	After 4 days of treat ment V PP PTT	After 10 days of treat ment V PP PTT	At the end of treat ment V PP PTT	Dura tion of hor mone treat ment	Rab bit No	Before hor mone treat ment V PP PTT	After 4 days of treat ment V PP PTT	After 10 days of treat ment V PP PTT	At the end of treat ment V PP PTT	Dura tion of hor mone treat ment
56	20	18	17	16	15	57	17	15	16	18	15	58	20	16	18	16	15
	38	25	27	19			24	23	26	27			34	29	31	25	
	43	35	32	35			26	40	35	38			32	44	42	43	
63	19	21	16	18	18	65	19	17	17	15	18	64	20	18	17	15	18
	44	29	28	36			24	19	29	23			36	22	29	27	
	36	39	33	34			23	33	38	31			40	46	41	36	
7	19	18	18	14	22	69	18	22	18	16	22	68	21	18	17	15	22
	3	30	30	24			30	31	30	31			31	29	25	28	
	43	39	37	26			32	36	39	34			45	56	43	50	
70	18	18	17	17	24	67	19	17	18	17	24	61	20	19	18	19	24
	24	30	31	29			25	29	24	28			39	32	35	30	
	37	40	46	37			29	42	18	35			31	34	37	31	
72	18	17	16	15	26	75	20	20	16	13	26	59	18	17	17	–	–
	29	27	26	22			24	26	26	21			26	22	30	–	
	34	39	51	27			37	34	36	28			43	43	40	–	
62	18	18	17	13	30	74	13	18	17	15	30	66	19	19	18	15	30
	25	26	27	24			24	23	24	30			2	22	32	21	
	28	44	41	33			41	34	34	38			29	35	42	37	
73	17	17	17	15	30	70	18	17	16	–	–	60	18	18	17	–	–
	23	26	27	26			29	24	23	–			30	21	31	–	
	31	37	32	41			35	45	40				35	24	43	–	
71	18	18	18	–	–												
	30	33	32	–													
	4[illegible]	37	37	–													

TABLE 2

Presence of Fibrin Microthrombi in Endotoxin Injected Rabbits and Controls

Group	Rabbit No	Survival (hours)	Endotoxin (mg)	Heart	Lungs	Spleen	Liver	Kidneys	Individual index
I	56	24	1.4	1	3	3	3	3	2.6
	63	24	2.8	1	3	3	2	1	2.0
	77	24	4.2	0	3	4	2	1.5	2.1
	76	24	5.6	0	1.5	4	2	0	1.5
	62	24	0	0	1	0	0	0	0.2
	73	24	0	0	0	0	0	0	0.0
II	65	3	2.8	0	4	3	3	3	2.6
	57	24	1.4	0	3	3	0	1	1.4
	69	24	4.2	0	3	3	1	1.5	1.7
	67	24	5.6	0	1.5	2	2	1	1.3
	74	24	0	0	1	3	0	0	0.8
III	64	1	2.8	0	3	0	2	0	1.0
	68	1	4.2	0	3	0	0	0	0.6
	58	3	1.4	0	2.5	0	3	2	1.5
	61	12	5.6	0	3.5	1	2	3	3
	66	24	0	0	0	0	0	0	0.0
IV	93	1	3.5	0	3	0	1	0	0.8
	78	1	4.9	0	2.5	0	2	2	1.3
	94	3	3.5	0	3.5	3	2	3	2.3
	92	6	3.5	1	3.5	3	3	4	2.6
	53	12	2.1	0	2.5	4	3	3	2.5
	95	24	0	0	0	3	0	0	0.6
	96	24	0	0	3	3	0	0	1.2
	97	24	0	0	1	3	0	0	0.8
V	52	1	4.9	0	3	3	0	0	1.2
	111	*3*	*1.4*	*0*	*0*	*0*	*1*	*0*	*0.2*
	82	6	7.0	0	2.5	0	1	1	0.9
	81	24	2.1	0	3	0	0	0	0.6
	51	24	2.8	0	2	2	1	0	1.0
	110	24	4.2	0	0	0	0	0	0.0
	112	24	4.9	0	2	3	2	0	1.4
	108	24	5.6	0	3	3	2	0	1.6
	113	24	0	0	0	3	0	0	0.6
	114	24	0	0	2	0	0	0	0.4
VI	104	6	2.8	2	3	3	3	4	3.0
	106	*6*	*2.8*	2	*4*	*3*	*3*	*4*	*3.2*
	105	12	2.8	0	3	4	3	4	2.8
	107	24	2.8	1	3	3	2	3	2.4

Group I rabbits pretreated with Enavid®. Group II rabbits pretreated with Enavid® and ACTH. Group III rabbits pretreated with Enavid® and chorionic gonadotrophin. Group IV pregnant rabbits. Group V non pregnant rabbits. Group VI non pregnant rabbits prepared by one previous endotoxin injection. 0 no thrombi. 1 one thrombus. 2 two to ten thrombi. 3 more than ten thrombi. 4 thrombi in almost every vessel in the tissue section. The average value from the five organs studied in each rabbit gives the individual index.

RESULTS

Changes in the investigated coagulation parameters during the pre parators hormonal treatment (groups I to III) are shown in *Table 1*

Individual variations in factor V PP and PTT before and during treatment were very broad and for this reason values are given in seconds and not in percentages During treatment factor V steadily increased PP and PTT oscillated about the original levels without any clear tendency

Changes in the coagulation parameters investigated during the 24 hours period following the provocatory endotoxin injection in groups I to VI are shown in *Figs 1 to 6* The curves for factor V PP and PTT represent individual rabbits in the case of endotoxin injected rabbits curves are given as unbroken lines in the case of non injected rabbits as broken lines Curves that do not cover the entire period represent rabbits dying spontaneously during the experiment Changes in the curves show to some extent the same tendency in all groups but not the same magnitude The observed times for factor V were much more prolonged in groups III and VI than in groups I II and V and the times for PP were more prolonged in groups III and IV than in the other groups The PTT values demonstrate the greatest change in groups V and VI with very marked prolongation in some rabbits The individual variations however are enormous in these groups

Modifications in the coagulation times seem to appear after the first hour following the endotoxin injection and reach a maximum 6 to 12 hours after injection in most rabbits In some of the rabbits in groups I II and V and in one rabbit in group VI the prolonged coagulation times in the 6th or 12th hour sample returned to normal levels in later samples a phenomenon which did not occur in groups III and IV A remarkable modification is seen in groups II IV and V curves of some of the rabbits showing a marked fall corresponding to a pronounced increase of the coagulation factors concerned in the ½ or 1 hour sample Comparison of individual curves shows rather good agreement as to the shape of the curves for factor V PP and PTT with the exception of PTT in group VI

Mortality was more pronounced in groups III IV and VI—12 out of 13 rabbits injected with endotoxin died during the 24 hours period following the injection—than in groups I II and V where 4 out of 12 endotoxin injected rabbits died spontaneously Six rabbits in groups I-III died spontaneously during the hormone treatment before receiving any injection of endotoxin 3 of pneumonia (nr 59 60 75) one with severe purulent otitis (nr 70) while two (nr 71 72) did not show any apparent cause of death

Presence of fibrin thrombi semi-quantified as previously described are shown in Table 2 The individual indexes correspond very well with the modification in the curves for factor V PP and PTT for individual

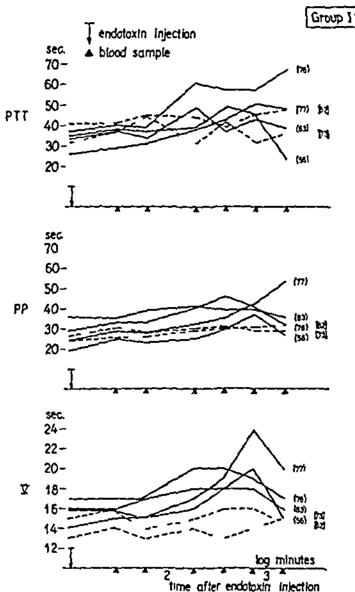


Fig 1

Modifications of factor V prothrombin proconvertin (PP) and partial thromboplastin time (PTT) during the 24 hours period following endotoxin injection in rabbits pretreated with Fnavid® Each curve represents one rabbit Endotoxin injected rabbits are given by unbroken line animal numbers are in round brackets Non endotoxin injected rabbits are given by broken line animal numbers are in square brackets

rabbits Control rabbits not injected with endotoxin have low indexes 0 0 to 1 2 Endotoxin injected rabbits have higher indexes depending on the experimental procedure and the survival time Rabbits in group VI (classical Shwartzman procedure) show the highest indexes rab

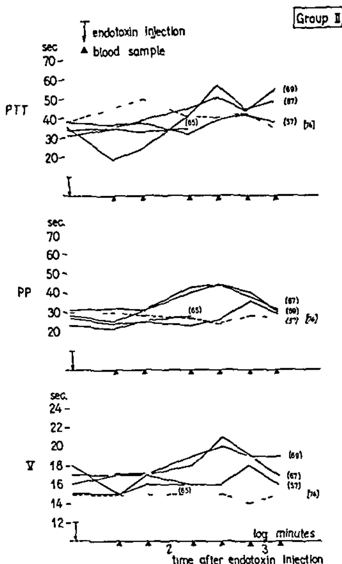


Fig ⁹

Modifications of factor V prothr mbin procon e tin (PP) and partial thrombo plastin *time (PTT) during the 24 hours period f ll) ving e dotoxin injecti)n in* rabbits pretreated with Enavid® and ACTH Fa h curve represents one rabbit. Endotoxin injected rabbits are given b unbroken lines nimal numbers are in round brackets Non endotoxin injected ral bit i g n by br ken line animal number is in quare bra k t

bits in group V (non prepared normal r ibbits) the lowest Intermediate index values are obtained in groups IV (pre gnant rabbits) I II and III (hormone pretreated rabbits) Only r ibbits surviving the endotoxin injection for at least 3 hours however h id individual ind x s above

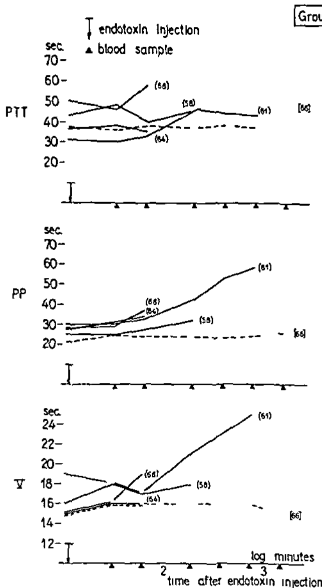


Fig 3

Modifications of factor V, prothrombin proconvertin (PP) and partial thromboplastin time (PTT) during the 24 hour period following endotoxin injection in rabbits pretreated with Enavid® and chorionic gonadotrophin. Each curve represents one rabbit. Endotoxin injected rabbits are given by unbroken lines, animal numbers are in round brackets. Non endotoxin injected rabbit is given by broken line, animal number is in square bracket.

the level of the controls. When individual indexes were high, fibrin thrombi were found disseminated in all organs. In the brain, however, fibrin thrombi appeared almost exclusively in the vessels of the choroid plexus. The incidence of fibrin thrombi in the organs were

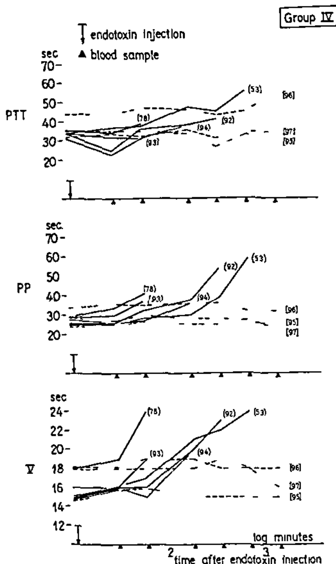


Fig 4

Modifications of factor V prothrombin proconvertin (PP) and partial thromboplastin time (PTT) during the 24 hours period following endotoxin injection in pregnant rabbits Each curve represents one rabbit Endotoxin injected rabbits are given by unbroken lines animal numbers are in round brackets Non endotoxin injected rabbits are given by broken line animal numbers are in square brackets

among the endotoxin injected rabbits lungs 93 per cent liver 84 per cent spleen 69 per cent kidneys 62 per cent and heart 21 per cent while among the non injected control rabbits lungs 56 per cent spleen 56 per cent liver kidneys and heart 0 per cent

Rabbits dying spontaneously during hormone treatment had indi

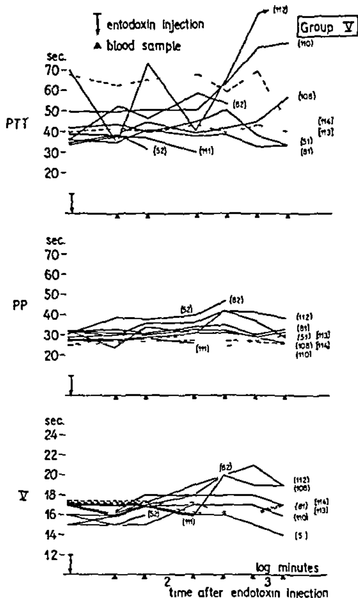


Fig 5

Modifications of factor V prothrombin proconvertin (PP) and partial thrombo plastin time (PTT) during the 24 hours period following endotoxin injection in non pregnant non pretreated rabbits Each curve represents one rabbit Endotoxin injected rabbits are given by unbroken lines animal numbers are in round brackets Non endotoxin injected rabbits are given by broken lines animal numbers are in square brackets

vidual indexes which did not exceed those of the control rabbits 0 6 to 1 2 Some fibrin thrombi however could be found in the liver and kidneys while control rabbits did not reveal any Rabbit nr 75 (group II) dying after 26 days of treatment with Enavid and ACTH of pneu

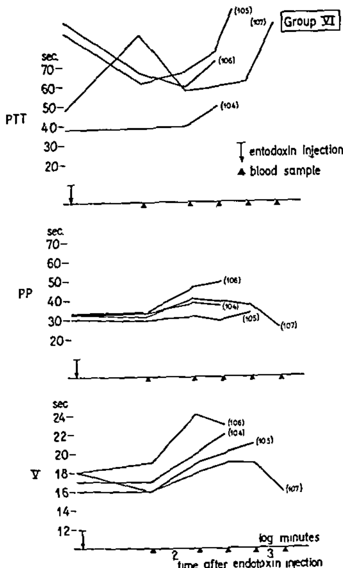


Fig 6

Modifications of factor V prothrombin proconvertion (PP) and partial thromboplastin time (PTT) during the 24 hours period following the second provocatory endotoxin injection in non pregnant rabbits Each curve represent one rabbit animal numbers are in round brackets

monia demonstrated the greatest modification in the PTT value (98 seconds) and among the six rabbits that died spontaneously it showed the highest individual index of fibrin thrombi (1 2)



Fig 7

Microthrombi in glomeruli from a rabbit (No 61) pretreated with Enavid® and chorionic gonadotrophin (Group III) given a single intravenous endotoxin injection Montage showing four different fields of the kidneys Phosphotungstic acid haematoxylin stain 100 ×



DISCUSSION

The modifications in factor X, PP and PTT observed following the injection of endotoxin are compatible with a consumption of coagulation factors due to intravascular clotting (*Muller Berghaus et al* 1963, 1964, *Krecke* 1964, *McKay* 1965) and histological examinations demonstrate that this phenomenon did in fact take place. Quantitatively the results of the coagulation analyses and the presence of fibrin thrombi agree very well, the higher the consumption of coagulation factors the greater the amount of fibrin thrombi in individual rabbits.

Previous work on the role of sex hormones administered alone failed to demonstrate any preparation for the GSR but the concomitant administration of two of these (estrogen and progesterone) actually prepared the rabbits for the reaction. Chorionic gonadotrophin alone in doses producing pseudopregnancy did not prepare for the reaction (*Cooper & McKay* 1960).

In the present experiment pretreatment with an oral contraceptive seemed to play a substantial role in the preparatory mechanism which was presumably enhanced by the addition of chorionic gonadotrophin to the pretreatment regimen but probably not by the addition of ACTH. A high similarity was found between pregnant rabbits and rabbits pretreated with an oral contraceptive plus chorionic gonadotropin regarding changes in the coagulation parameters studied and mortality after endotoxin injection and at least one rabbit (group III) survived long enough (12 hours) to develop the full blown picture of glomerular microthrombosis pathognomonic of the GSR (Fig 7).

SUMMARY

Changes in the blood coagulation parameters studied, factor X, PP and PTT, following the intravenous injection of a single sub lethal dose of endotoxin (E coli lipopolysaccharide) to female rabbits pretreated with an oral contraceptive alone or combined with ACTH or chorionic gonadotrophin are compatible with a consumption of these factors as a result of intravascular coagulation as this was histologically demonstrated by the presence of fibrin thrombi in several organs including the kidneys.

This consumption coagulopathy was closely correlated to the severity of the intravascular clotting, the higher the former the more disseminated the latter. These changes in rabbits pretreated with hormones followed by a single endotoxin injection were compared with those occurring in pregnant rabbits and in non pretreated non pregnant female rabbits following the intravenous injection of the same endotoxin and finally with those occurring in rabbits during the generalized Shwartzman reaction induced by the classical procedure.

The results suggest that preparation for the generalized Shwartzman reaction can be obtained in female rabbits by the administration

of an oral contraceptive alone or most probably to a greater extent combined with chorionic gonadotrophin Further investigations must however be performed before definite conclusions can be drawn

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SUMMARY

Changes in the blood coagulation parameters studied factor X PP and PTT following the intravenous injection of a single sub lethal dose of endotoxin (E coli lipopolysaccharide) to female rabbits pretreated with an oral contraceptive alone or combined with ACTH or chorionic gonadotrophin are compatible with a consumption of these factors as a result of intravascular coagulation as this was histologically demonstrated by the presence of fibrin thrombi in several organs including the kidneys

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The results suggest that preparation for the generalized Shwartzman reaction can be obtained in female rabbits by the administration

Similar observations were made by *Moore* (1935) *Rich* (1935) and *Franks* (1954)

However *Gaynor* (1938) *Kahler* (1939) *Howald* (1948) and *Karube* (1961) did not find any relationship between atrophy and neoplasia

In experimental carcinogenesis by chemical agents the sequence of histological changes seems to be damage to and death of cells followed by regeneration and tumour formation Similarly a relationship has been demonstrated experimentally between atrophy and cancer in many organs of the rat (see review by *Liavag* 1967)

The aim of the present investigation was to study the relationship between three processes occurring in the adult human prostate *atrophy* small alveolar proliferation interpreted as *regeneration* and *carcinoma*

MATERIAL AND METHODS

The material consists of the prostates from 340 consecutive autopsies of male individuals at the age of 40 years or above from the Department of Pathology Ullevål Hospital The bodies were kept at a temperature of 4 to 6 C and autopsy was usually performed 12 to 24 hours after death After fixation in 10 per cent formalin for 8 to 10 days the prostates were cut transversally in about 4 mm thick slices perpendicular to the long axis of the urethra Three slices from each gland were taken for microscopic examination viz one slice from the middle of the infracollicular part of the prostate (I) one from the middle of the collicular (II) and one from the middle of the supracollicular part (III)

The selected slices were embedded in paraffin and total transverse sections were cut on a microtome with the setting on 5 microns One section from each of the three selected slices was mounted stained with haematoxylin and eosin (H+E) and studied in the light microscope

PRESENT FINDINGS

Based on the criteria mentioned in a previous study (*Liavag* 1967) 90 (26 5 per cent) clinically unsuspected prostatic carcinomas (latent carcinomas) were found Most of the carcinomas were small and this is a prerequisite for the present study However three of the tumours were too large and destructive and they were excluded from the present consideration

Atrophy was graded as *slight* *moderate* or *marked* according to the severity of the microscopic findings (see *Liavag* 1967) Atrophy was found in 100 per cent of the prostates with carcinoma (Table 1) Among the prostates without carcinoma (Table 2) atrophy was also very frequent (90 3 per cent) but the figure was significantly higher for the carcinomatous prostates (χ^2 = 7 68 with 1 degree of freedom $P < 0\ 01$)

Generalized atrophy of the prostate was found in only 4 cases In the remaining cases atrophy was localized

Unsuspected prostatic carcinoma increased in frequency with age from 8 per cent in the 5th decade to 46 7 per cent in the 9th decade Similarly there is a trend for atrophy to increase with age (Table 2)

and the frequency was significantly higher for the patients over the age of 70 than for those under this age ($\chi^2 = 4.07$ with 1 degree of freedom $P < 0.05$)

TABLE 1

Occurrence of Prostatic Atrophy among the 87 Patients with Unsuspected Carcinoma of the Prostate

Age	Unsuspected carcinoma	Slight atrophy	Moderate atrophy	Marked atrophy	Atrophy All cases
40–49	2	1	0	1	2
50–59	11	1	1	9 (8[illegible] %)	11 (100 %)
60–69	28	0	6	22 (79 %)	28 (100 %)
70–79	24	0	3	21 (88 %)	24 (100 %)
80–89	20	0	1	19 (95 %)	20 (100 %)
90–99	2	0	0	2	2
Total	87	2 ([illegible] %)	11 (13 %)	74 (85 %)	87 (100 %)

TABLE 2

Occurrence of Prostatic Atrophy among 237 Patients without Prostatic Carcinoma

Age	Without carcinoma	Slight atrophy	Moderate atrophy	Marked atrophy	Atrophy All cases
40–49	23	3	5	11 (48 %)	19 (83 %)
50–59	43	8	10	19 (44 %)	37 (86 %)
60–69	90	10	30	40 (44 %)	80 (89 %)
70–79	59	7	20	30 (51 %)	57 (97 %)
80–89	21	1	4	15 (71 %)	20 (95 %)
90–99	1	0	0	1	1
Total	237	29 (12 %)	69 (29 %)	116 (49 %)	214 (90 %)

Atrophy was more severe in carcinomatous than in non carcinomatous prostates (Tables 1 and 2) Statistically the carcinomatous and non carcinomatous prostates showed a highly significant difference of atrophy in the three degrees slight moderate and marked ($\chi^2 = 25.87$ with 2 degrees of freedom $P < 0.00001$) The difference was also of about the same magnitude in all age groups Both in carcinomatous (Table 1) and in non carcinomatous (Table 2) prostates there is a trend for marked atrophy to increase in frequency with age

Microscopically the epithelium of atrophic glands appeared at first sight to be inactive But on closer examination it was seen that in one or more places of these glands there were often aggregations of cells indicating growth activity The nucleus was large the nucleolus prominent and the cytoplasm abundant compared with the atrophic cells

(Figs 1 and 2) The active looking cell aggregations often formed solid buds (Fig 1) which protruded into or through the fibrous stroma which surrounded the atrophic glands Small alveolar glands embedded in a stroma of loose connective tissue were seen scattered or crowded around the atrophic ones (Figs 2 and 3) The findings suggested that the small alveolar proliferations originated from the buds or active looking cell aggregations of atrophic glands The newly formed alveoli *could be quite regular* (Fig 2) or they were highly irregular both with regard to the acini and the single cells (Fig 4)

The small alveolar proliferations connected with atrophic glands displayed a structure and differentiation similar to the glandular pattern of the immature prostatic epithelium of infants and of small alveolar carcinoma Immaturity with little or no secreting activity was characteristic of the cells in all the three structures mentioned and they showed a similar pattern with formation of small acini (Figs 5 6 and 7)

TABLE 3

Occurrence of Small Alveolar Proliferation in 87 Prostates with Unsuspected Carcinoma.

Age	Unsuspected carcinoma	Slight proliferation	Moderate proliferation	Marked proliferation	Proliferation All cases
40–49	2	0	0	1	1
50–59	11	2	1	8 (73 %)	11 (100 %)
60–69	28	0	8	18 (64 %)	26 (93 %)
70–79	24	2	4	16 (67 %)	[illegible] (9[illegible] %)
80–89	20	1	5	13 (65 %)	19 (9[illegible] %)
90–99	2	0	1	1	2
Total	87	5 (6 %)	19 ([illegible] %)	57 (66 %)	81 (93 %)

TABLE 4

Occurrence of Small Alveolar Proliferation in 237 Prostates without Prostatic Carcinoma

Age	Without carcinoma	Slight proliferation	Moderate proliferation	Marked proliferation	Proliferation All cases
40–49	23	2	3	10 (43 %)	15 (6[illegible] %)
50–59	43	[illegible]	5	16 (37 %)	27 (63 %)
60–69	90	[illegible]	18	[illegible]0 (2[illegible] %)	60 (67 %)
70–79	59	17	11	16 ([illegible]7 %)	44 (75 %)
80–89	21	4	6	9 (43 %)	19 (91 %)
90–99	1	1	0	0	1
Total	237	5[illegible] (2[illegible] %)	43 (18 %)	71 (30 %)	166 (70 %)

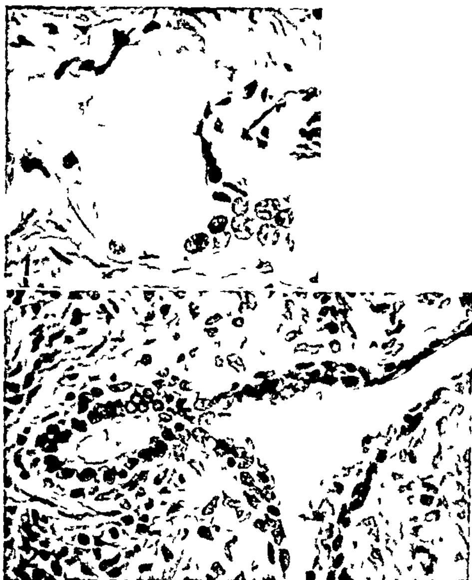


Fig 1 Atrophic acinus with aggregation of cells forming a solid bud Note the greatly enlarged nuclei and the prominent nucleoli (No 147 II) × 530 H + E

Fig 2 Small alveolar acinus to the left budding from atrophic duct Note the cells with large nuclei (No 200 I) × 530 H + E

The small alveolar proliferation was graded as *slight moderate* or marked according to the number of acini (see *Havag* 1967)

The occurrence of small alveolar proliferation in the prostates with carcinoma (93 1 per cent Table 3) was significantly higher than the corresponding figure for the non carcinomatous prostates (70 per cent Table 4) (X^2 = 17 44 with 1 degree of freedom $P < 0\ 001$) There is a



Fig 3 Marked small al e lar proliferatic n an t l with atr phic duct (No 1f7 II) × ?10 H + I

Fig 4 Irregul r small alveolar prolife ti n t tl l ft nnected with smaller atrophic duct to the right (No 1 II p ;l) z e) × 530 H + F

trend for the small alveolar prolif ation to in re ise with age (Table 4) and the frequency was significantly higher for the patients over the age of 70 than for those under this age (X^2 = 4 72 with 1 degree of freedom P < 0 05)

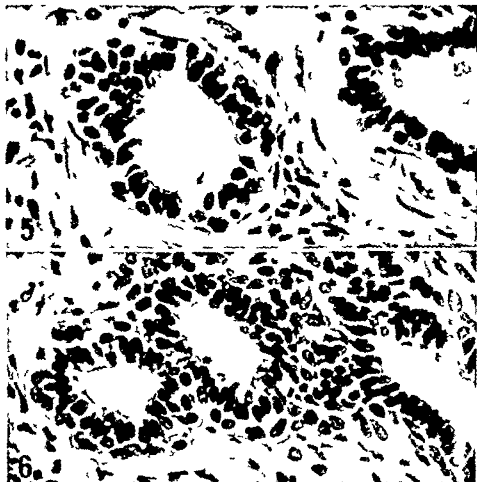


Fig 5 The immature feature of a small alveolar proliferation End of atrophic duct to the right (No 1C9 I Man 59 years old) × 530 H + E

Fig 6 Glandular feature of normal immature prostatic epithelium Duct to the right (No 1531/61 Boy 2½ years old) × 530 H + E

Small alveolar proliferation was of greater degree among the carcinomatous prostates than among the non carcinomatous ones (Tables 3 and 4) Statistically the carcinomatous and the non carcinomatous prostates showed a highly significant difference in small alveolar proliferation by the three degrees slight moderate and marked (χ^2 = 23 05 with 2 degrees of freedom $P < 0\,001$) The difference was also of about the same magnitude in all age groups

In 40 prostates (46 per cent Table 5) atrophy small alveolar proliferation and carcinoma were found side by side and/or intermingled in the same area of a microscopic section (Fig 8) In 32 prostates (36 8 per cent Table 5) the small alveolar proliferations connected with atrophic glands were morphologically indistinguishable from the acini of the surrounding carcinoma (Figs 7 and 9)

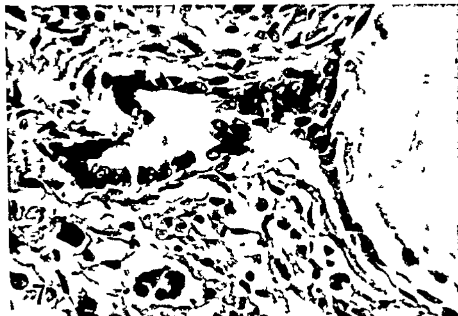




Fig 7 Cells which are morphologically indistinguishable from those of the surrounding carcinoma are budding off from an atrophic acinus containing a corpus amylaceum (No 125 II) × 530 H + E

Fig 8 Marked atrophy and small alveolar proliferation (left) in same area as carcinoma (right) (No 167 II) 3 H + E



Fig 9 Marked atrophy and fibrosis of a whole lobule A small alveolar carcinoma seems to originate from the terminal branches of the atrophic duct (No 239 II) × 85 H + E

TABLE 5

Relationship between Atrophy small Alveolar Proliferation and Carcinoma

Histological findings	Number of prostates
Atrophy and small alveolar proliferation found separated from area of carcinoma	9
Atrophy and small alveolar proliferation found within same area as carcinoma	40
Small alveolar proliferations connected with atrophic glands were morphologically indistinguishable from the surrounding carcinoma	32
Atrophy and carcinoma but no small alveolar proliferation	6
Total number of prostates with unsuspected carcinoma	87

DISCUSSION

The prostatic epithelium has a secretory cycle (*Petersen* 1909) and according to this there are functional variations in the size of the cells. The actively secreting cells are columnar while the resting cells are cuboidal or even flat. Cells in the resting phase may therefore be difficult to distinguish from atrophic cells. However, the secretion of the prostate is continuous (*Petersen* 1909, *Stieve* 1930) and it is not likely that all cells of even one acinus are secreting synchronously. Furthermore, structural changes accompany the atrophy, such as disappearance of the intra-acinar cellular crests and papillae, dilatation, shrinkage and finally disappearance of acini and ducts, reduction of smooth muscle and increasing fibrosis. Usually it is therefore possible to distinguish between resting and atrophic epithelium.

Atrophy leads to death of cells and disappearance of glandular structures (*Reischauer* 1925, *Moore* 1936). Further, the small alveolar proliferations were found only in connection with atrophic glands. Because regeneration is initiated by reduction of the total mass of a cell population (*Hartmann* 1928, *Weiss* 1955, *Weiss & Kavanau* 1958) it is therefore suggested that the small alveolar proliferations represent a regenerative mechanism. It is also in accordance with the definition of regeneration (*Payling Wright* 1958) that the small alveolar proliferations represent a "*replacement growth*" which takes place from the "*survivors*" of cells in atrophic glands. Furthermore, it is characteristic of regenerative growth that it displays a structure and differentiation which is essentially similar to the corresponding immature tissue of infants (*Willis* 1958). The small alveolar proliferations also fulfil this criterion of regeneration.

On the other hand, it has not been shown that the small alveolar proliferations mature into normal acini (*Liavåg* 1967). The incomplete or failing differentiation may therefore raise doubt whether the small alveolar proliferations represent true regeneration since they do not lead to re-establishment of normal conditions. However, it has been maintained both by *Buchner* (1966) and by *Letterer* (1959) that the new-formed tissue of regeneration does not always re-establish normal mass and structure.

There is thus considerable evidence in support of the view that the small alveolar proliferations are the result of a regenerative process. It should be emphasized, however, that the findings do not exclude other mechanisms of these cellular proliferation.

As previously mentioned, certain cytological and structural signs indicate that growth takes place from active-looking cell aggregations in atrophic glands. By means of colcemid it has also been possible to demonstrate mitoses in atrophic glands (*Liavåg* 1967).

It is suggested that atrophy gives rise to the small alveolar proliferations which in turn may develop into carcinoma. Statistically

there was a significant correlation between the three processes, and it is therefore unlikely that they co-exist merely by chance.

Atrophy could be secondary to carcinoma. This is unlikely, however, because the carcinomas were usually too small to cause any significant atrophy by infiltration and compression.

The budding of cells from atrophic glands indicates an active growth process in which atrophy and small alveolar proliferation represent either steps in the development of carcinoma or the three processes may be independent manifestations of a common underlying cause. Because the small alveolar proliferations connected with atrophic glands are often morphologically indistinguishable from the surrounding carcinoma (Figs 7 and 9), it seems most likely that the three processes represent phases in the development of malignancy.

The inception of malignancy is thought to take place during the process of atrophy ("initiating phase") and growth to be induced by a regenerative stimulus with the formation of undifferentiated acini and ultimately frank carcinoma ("promoting phase"). The same sequence of histological changes are observed in several organs in experimental carcinogenesis produced with chemical agents (*Glinos & Bucher* 1949, *Glinos et al.* 1951, *Daoust* 1962, *Iversen & Evensen* 1962, *Vasiliev et al.* 1962, *Daoust & Molnar* 1964).

The process of atrophy involves long continued growth depression and cell damage, possibly resulting in the development of a new and irreversibly changed cell type in response to the unfavourable conditions (cf. *Haddow* 1947).

Generalized atrophy of the prostate, however, involving all epithelial structures seemed not to be compatible with the development of carcinoma, because in 86 of the carcinomatous prostates the atrophy was localized and combined with hyperplastic and/or residual normal glandular tissue. The 87th prostate was previously operated on for benign hyperplasia. Thus it appeared that carcinoma developed only in prostates with localized atrophy in which signs of function and growth persisted.

SUMMARY

The relationship between atrophy, small alveolar proliferation (regeneration) and carcinoma of the prostate was studied in consecutive autopsy material of 340 males at the age of 40 or above.

Small alveolar proliferations connected with atrophic glands seemed to originate from active looking cell aggregations or buds in the atrophic glands. It is suggested that the small alveolar proliferations fit into a regenerative mechanism in the way that they represent a replacement growth which takes place from survivors of cells in atrophic glands.

Statistically there was a significant correlation between atrophy

small alveolar proliferation and carcinoma The inception of malig nancy is thought to take place during the process of atrophy and growth to be induced by a regenerative stimulus with the formation of undifferentiated acini (small alveolar proliferations) and ultimately frank carcinoma

It appeared that carcinoma only developed in prostates with localized atrophy in which signs of function and growth persisted

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MATERIAL AND METHODS

33 human corpses of both sexes and 4 surgical biopsies obtained from female subjects were used in the study. The time interval between the moment of death and the moment of excision, the ambiant temperature as well as the cause of death and the age of the subject were noted.

The different time intervals varied between 35 min and less than 6 days. In 5 cases the time of death was not known with certainty. Three different grades of ambiant temperature were established: over 18 C, about 15 C and about 4 C. The causes of death were classified into three main groups: natural, traumatic and toxic. The age varied from 3? to 83 year.

A skin sample (X 1 cm) was taken from the axilla. One part of each sample was fixed in 4 per cent neutral buffered formaldehyde for 24 hours, embedded in paraffin and sectioned at 6 microns. Sections were stained with haematoxylin eosin and alcoholic safran. Two other parts were fixed in icecold formol calcium, the first of these to be used for the study of alkaline phosphatase was fixed for 10 min, the second to be used for the study of acid phosphatase and AS esterases for 4 hours. Both were stored in a solution of gum arabic sucrose at 4 C. Frozen sections were cut at 6 microns by the Jung Frig mat. The naphtol AS BI phosphate technique (*Burstone* 1962) was performed at room temperature to demonstrate the presence of alkaline phosphatase and at 37 C for the acid phosphatase. The non specific esterases were demonstrated by the Naphtol AS acetate technique (Gomori method modified by *Pearse* 1960).

The enzyme activity was estimated in each tissue structure in 2 different ways. The first, a semi quantitative method, was based on the time of appearance of the histochemical reaction. This estimation was performed using a time fractioned incubation: the incubation for alkaline phosphatase was interrupted after 30 sec, 1 min, 2 min, 6 min, 12 min and 30 min respectively, for acid phosphatase after 1, 2, 4, 8, 15, 30 and 40 min, and for non specific esterases after 30 sec, 1 min, 2 min, 6 min, 10 min and 50 min. The second was estimated on the basis of the amount of the final reaction product after the maximal incubation time using an arbitrary scale 0 to 2 +.

RESULTS

Histological Changes

In most of the samples no morphological changes could be noted. Only in 2 cases of putrefaction in which intervals of 74 h and no less than 6 days respectively were noted, cellular changes were observed in all tissue structures. These changes consisted principally in a loss during staining of nuclear detail and a detachement of the cytoplasm from the nuclear membrane with the appearance of a perinuclear halo.

Enzymes

All the results were analysed statistically. Groups were made up in various ways as related to the factor investigated. In the case of the qualitative method the distribution of the χ^2 was studied, in the case of the semi quantitative method the cumulative F distribution was used.

A considerable activity of alkaline phosphatase was found in the endothelial cells of the blood capillaries and a lesser amount in the epithelial cells of the apocrine and eccrine glands. No activity was observed in the other structures.

The activity of acid phosphatase was pronounced in the granular layer of the epidermis and in the epithelial cells of the eccrine and



Fig 1

Non specific AS esteras es activity in the axilla skin 58 year old male Sample is taken 1 h postmortem Stro g activity in the epidermis and the fibrocytes of the dermis (naphtol AS acetate method of G m ri Pearse no nuclear c unterstain × 250)



F

Acid ph sphatase activity in the apocrin f the axilla a) 66 year cl f m l Samp l taken 2 h 10 p stm rtem b 73 male in state of advanced p t f tion Strong activity in both cases sl ght tu ion in case b (Napht ph s phate technique f Bur tone n u lear c unterstain × 50

A) *Effect of the Time Interval between Death and Excision*

	Granular layer	Malpighii L.	Inner hear sheat	Sebaceous gl
Alk Pase	Absent	Absent	Absent	Absent
Ac Pase	Qual homog Semiquant homog	Qual homog Semiquant homog	Qual homog Semiquant homog	Qual homog Semiquant homog
A S Est	Absent	Qual homog Semiquant homog	Qual homog Semiquant homog	Qual homog Semiquant homog

B) *Effect of Ageing*

	Granular layer	Malpighii L	Inner hear sheat	Sebaceous gl
Alk Pase	Absent	Absent	Absent	Absent
Ac Pase	Qual homog Semiquant homog	Qual homog Semiquant homog	Qual homog Semiquant homog	Qual homog Semiquant homog
A S Est	Absent	Qual homog Semiquant homog	Qual homog Semiquant homog	Qual homog Semiquant homog

C) *Effect of the Cause of Death*

	Granular layer	Malpighii L	Inner hear sheat	Sebaceous gl
Alk Pase	Absent	Absent	Absent	Absent
Ac Pase	Qual homog Semiquant homog	Qual homog Semiquant homog	Qual homog Semiquant homog	Qual homog Semiquant homog
A S Est	Absent	Qual homog Semiquant homog	Qual homog Semiquant homog	Qual homog Semiquant homog

The following abreviations are used Qual qualitative method Semi quant semi quantitative method Homog homogeneous The knowledge homogeneous means that no difference between the experimental groups is observed

1
Statistical Analysis

Apocrine gl	Eccrine gl	Blood vessels	Fibrocytes
Qual $0.5 < P < 0.9$ Semiquant homog	Qual homog Semiquant homog	Qual homog Semiquant $0.05 < P < 0.1$	Absent
Qual homog Semiquant homog	Qual homog Semiquant homog	Absent	Qual homog Semiquant $0.05 < P < 0.025$
Qual homog Semiquant homog	Qual homog Semiquant homog	Absent	Qual homog Semiquant homog

Apocrine gl	Eccrine gl	Blood vessels	Fibrocytes
Qual homog Semiquant homog	Qual homog Semiquant homog	Qual homog Semiquant homog	Absent
Qual homog Semiquant homog	Qual homog Semiquant homog	Absent	Qual homog Semiquant homog
Qual homog Semiquant homog	Qual homog Semiquant $0.5 < P < 0.025$	Absent	Qual homog Semiquant homog

Apocrine gl	Eccrine gl	Blood vessels	Fibrocytes
Qual homog Semiquant homog	Qual homog Semiquant homog	Qual homog Semiquant homog	Absent
Qual homog Semiquant homog	Qual homog Semiquant homog	Absc	Qual homog Semiquant $P > 0.05$
Qual homog Semiquant homog	Qual homog Semiquant homog	Ab en	Qual homog Semiqu nt homog

3

apocrine glands In the latter the activity was seen as a nodular luminal accumulation of the final reaction product The activity was modest although appreciable in Malpighi s layer the inner hair sheat the glandular cells of the sebaceous glands and in the fibrocytes of the dermis Other cells and structures were not investigated systematically but showed practically no activity

As esterase activity was also observed as a nodular reaction in the epithelial cells of the apocrine glands and as a more diffuse cytoplasmic reaction in the eccrine glands and in the fibrocytes Appreciable amounts were observed in Malpighi s layer of the epidermis (Fig 1) the inner hair sheat and in the glandular cells of the sebaceous glands Other cells and structures were not investigated

Most often any decrease in the activity of the 3 enzymes could not be observed immediately after death and such decrease was not observed either in the postmortem period studied (30 min to 72 h) (Fig 1 Fig 2a) the groups made up in relation to the time interval were homogeneous Nevertheless the results of calculation were bordering on signification in the case of alkaline phosphatase of the apocrine gland expressed qualitatively in the case of the blood vessels expressed semi quantitatively and in the case of acid phosphatase of the fibrocytes expressed semiquantitatively (Table 1 A) On the other hand in one of the cases of putrefaction acid phosphatase and AS esterases were found to be diffused out of their natural site in the cytoplasm of the epidermis cells and epithelial cells of the apocrine and eccrine glands (Fig 2b) Meanwhile acid phosphatase was completely absent in the hair sheats and in the sebaceous glands

Similarly the activity did not differ in the groups constituted according to the temperature of the corpse and the effect of ageing In this last study also a borderline calculation in the case of AS esterases of the eccrine glands expressed semiquantitatively was noted (Table 1 B)

Finally the only significant difference observed concerned the activity of acid phosphatase in the fibrocytes related to the cause of death The mean incubation time was shorter in the group of natural deaths than in the group of traumatic deaths 17 to 28 min Among the other structures no differences according to the cause of death could be found (Table 1 C)

COMMENT

In general this study shows that the 3 hydrolytic enzymes in skin and skin appendages are very stable and resistant to postmortem autolysis at least until putrefaction of the body No significant influence of body temperature and ageing of the subjects could be shown Thus the results state precisely and confirm amply the findings in this field by *Raekallio & Fatteh*

However this general rule has one exception the activity of acid

phosphatase in the fibrocytes of dermis measured by the semi quantitative method is significantly lower in cases of traumatic death than in cases of natural death This difference cannot be related to the age of the subject (cfr supra) and there is actually no satisfactory explanation of this interaction Further enzyme cytochemical studies (especially the oxydative enzyme systems) may serve to approach the settlement of the problem

Furthermore the results are doubtful in a few cases and more thorough investigations must be undertaken before definite conclusions in this matter can be drawn These results are the effect of the time interval between death and excision on the alkaline phosphatase activity of the apocrine and eccrine glands and on the acid phosphatase of the fibrocytes the effect of ageing of the subject on the AS esterases activity of the eccrine glands

SUMMARY

The effect of postmortem autolysis and other related variations on the histochemical reactions of alkaline phosphatase acid phosphatase and AS esterases of the axilla skin and appendages have been studied in 33 human bodies of both sexes 4 surgical biopsies obtained from female subjects were used as controls The time interval between the moment of death and the time of excision varied from 30 min to less than 6 days

Three different grades of ambiant temperature were established over 18 ±15 and ±4 C The causes of death were classified into 3 categories natural traumatic and toxic The ages of the subjects varied from 32 to 83 years Most often the 3 enzymes were remarkably stable and the activity in control and autopsy samples was not found to differ In the same way there was no decrease as regards the lengthening of the time interval On the other hand in one case of putrefaction the enzymes had diffused out of their natural sites With a view to the temperature of the body the effect of ageing and the cause of death no difference in activity was found

Nevertheless (considering the relation between the enzyme activity and the cause of death) in the groups natural death and traumatic dath the content of acid phosphatase in the fibrocytes of the dermis was found to differ significantly the mean incubation time of histochemical reaction was markedly longer and thus the enzyme activity lower in the second group There is actually no satisfactory explanation of this interaction Furthermore in a few studies the results of statistic calculation were borderline cases effect of the time interval on the alkaline phosphatases content of the apocrine and eccrine glands and on the acid phosphata content of the fibrocytes and effect of ageing on the AS esterases content of the eccrine glands

Further investigations must be undertaken if definite concl s ons in these particular fields are to be drawn but the gener l conclusion

seems to be that these hydrolytic enzymes do not constitute a favorable medium for the evaluation of the time of death

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TYPES OF CONTACTS IN VITRO BETWEEN ROUS MOUSE TUMOUR CELLS AND CHICKEN FIBROBLASTS AS STUDIED WITH THE ELECTRON MICROSCOPE

By

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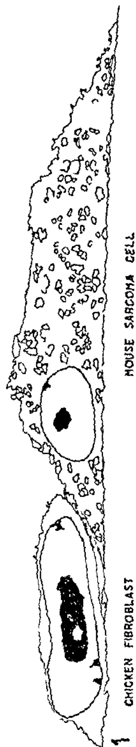
Received 9 i 68

In several oncogenic virus tumour systems there are indirect indications of the retention of at least part of the viral genetic material in the tumour cells (new transplantation antigens T antigens) even after long term serial passages *in vitro* and *in vivo* and in spite of the fact that no release of virus can be detected Direct proof can be obtained in the SV40 and mammalian Rous tumour systems by co cultivation *in vitro* of non virus releasing tumour cells with susceptible indicator cells whereby the viral genome is induced to release infectious virus (*Gerber & Kirschstein* 1962 *Šimkovic et al* 1962 *Svoboda* 1964 *Jonsson* 1966) In the Rous system these findings correspond *in vivo* with a formation of virus releasing chicken tumours after the inoculation of mammalian tumour cells (*Svoboda* 1960 & 1961 *Ahlstrom & Forsby* 1962 *Ahlstrom & Jonsson* 1962)

It is fairly well established that most Rous tumours induced in mammalian species do not release infectious virus neither *in vitro* nor *in vivo* (*Ahlstrom & Forsby* 1962 *Ahlstrom & Jonsson* 1962 *Svoboda et al* 1963 *Jonsson* 1966) Some evidence has however been presented that mammalian cells may be capable of synthesizing chicken tumour virus (*Svoboda* 1964 *Altaner & Svec* 1966) Virus particles have never been observed in ultrastructural studies of serially transferred Rous rat tumour cells (*Lindberg* 1968 to be published) Co propagation of Rous mouse tumour cells with susceptible chicken cells however results in the transformation of the chicken cells and the release of extracellular infectious virus Obviously an intim contact between the two cell types is necessary for the exchan of material required for virus synthesis

This work was supported by grants C 415 from *NIH* and 66 5   m the *Swedish Cancer Society*




CHICKEN FIBROBLAST
MOUSE SARCOMA CELL
1




MOUSE SARCOMA CELL
CHICKEN FIBROBLAST
2

This work was performed in an attempt to study such contacts at the ultrastructural level

MATERIAL AND METHODS

The mouse tumour cells were derived from serially isografted sarcomas originally induced by subcutaneous inoculation of Rous chicken sarcoma (SR-RSV) into new born mice of the F_1 hybrid AxC57Bl/Kl After 15 passages *in vivo* two tumours designed RS57A and RS57D were explanted *in vitro* and serially propagated as cell cultures in Eagle s basal medium containing 6 per cent calf serum in humidified atmosphere containing 5 per cent carbon dioxide

Tumour cell suspensions were prepared by trypsinization of cell cultures and the number of trypan blue unstained tumour cells was estimated (*Boyse et al* 196?) after irradiation of the suspension

Irradiation procedure In order to prevent multiplication and overgrowth of tumour cells in the mixed cultures the tumour cell suspensions were irradiated at 0 C by X rays generated at ?00 kV 15 mA and filtered by 1 mm Al The total X ray dose was 8 000 r

Chick embryo cultures Primary cultures of 9–12 day old white Leghorn chicken embryo cells were prepared in 100 mm petri dishes (Falcon) according to *Temin & Rubin* (1958) Eagle s medium with tryptose phosphate and 8 per cent calf serum was used with the addition of antibiotics

Mixed cultures were prepared in 100 mm petri dishes by seeding 1 5 × 10⁶ chicken embryonic fibroblasts (CEF) and 2 5 × 10 irradiated Rous mouse tumour cells After outgrowth of the CEF for 4 to 5 days the cultures were passed to 60 mm petri dishes which were covered with agar med um on the following day

Ultrastructural studies The procedure follows essentiall that described by *Heyner* (1963) and *Sutton* (19(5) with minor modifications

At the time of readily demonstrable transformation of the CFF (6 to 9 days after the passage) the agar covering was removed and the cell layer was immediately fixed by the careful pouring of cold 3 per cent glutaraldehyde in saline (pH adjusted to 7 2) into the dish After 60 minutes and repeated washings the cell layer was postfixed in 1 per cent OsO s-collidine buffer for 60 minutes (*Wood & Luft* 19(5) After washing the cell layer was dehydrated in graded alcohols and embedded in Epon 812 according to *Luft* (1961) with the omission of the intermedient solvent propyleneoxide to avoid dissolution of the plastic material The unpolymerized solution was mixed and poured directly into the petri dish giving an about 2 mm thick plastic layer on top of the cells After polymerization under gradually in creased temperature the plastic material of the petri dish could easily be removed from the slice of Epon The embedded cell layer showed good preservation of mor phology In most cases the cell layer was stained sufficiently to be studied under the light microscope but sometimes the contrast was incre sed by using ? per cent phosphotung tic acid in 70 per cent alcohol in the dehydration series

Parts of the embedded cell layer containing a single tumour cell and closely adjacent CFF were cut out with scissors and the pecimen trimmed to a pyramid like

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Figs 1–

Fig 1 Survey picture of a normal chicken fib oblast and an irradiated Rous mouse tumour cell The fibroblast is flatte d with n el ngated slender nucleus while the tumour cell has a more unded f rm and an almost spherical nucleus Note the difference of cyt pl mi ontent with numerous small vesicles in the tumour cell Se t n thickne was working at 500 Å × 2 800

Fig 2 Survey picture of an intimate cont t bet een a Rous mouse sarcoma cell an l a chicken fibroblast The nu l f the tumour cell is sectione l tan gentially In the chicken fibrobla t re is end plasmatic reticulum which is hardly ever een in the irradia e l tum ur cells The arr ws indicate the fusion zone between the t o cells S tion thickness" was working t 400 Å × ? 100

shape with the cells as close to the top as possible Sectioning was performed on a LKB Ultrotome at right angle to the former Epon plastic dish interface Small remnants of plastic material sometimes caused difficulties in sectioning The section thickness was therefore usually set slightly above (700 800 A) the normal range but sometimes good sections were obtained at 400 A

Further processing of the specimen was made in the conventional way The examination took place in a Zeiss EM9 electron microscope equipped with a condensor

RESULTS

Even under the light microscope the surviving radiation damaged Rous mouse tumour cells can be recognized in the mixed cultures and usually distinguished not only from untransformed but also from transformed CEF (*Jonsson* 1966) Under the electron microscope several differences make such a distinction possible The cytoplasm of the irradiated Rous mouse tumour cell contains numerous small or medium sized vesicles (Figs 1–2) The tumour cell usually has a more rounded appearance The nucleus is rounded or sometimes has an almost spherical form which is in sharp contrast to the elongated chicken fibroblast with its long slender nucleus (Fig 1) Even after transformation the fibroblast usually retains its flat appearance on the culture dish Membrane bound cell organelles are extremely rare in the tumour cells cultivated *in vitro* while the cytoplasm of the chicken fibroblasts always contains a more or less well developed coarse endoplasmatic reticulum coated with ribosomes In the transformed chicken fibroblasts there are always clearly demonstrable remnants of endoplasmatic reticulum

Studies in the electron microscope of the relation between fibroblasts *in vitro* usually reveal a small space between the outer cell membranes of adjacent cells (*Easty & Mercer* 1960)

This also applies to our mixed cultures containing cells of different origins (Fig 3) in which this space has a width of 0 1–0 5 μ The surface of both types of cells is covered with microvilli and often such microvilli from one cell come into close contact with the microvilli or outer cell membrane of another As a rule there are no abnormalities or interruptions in the cell membranes of the two cells and their outlines can be followed continuously (Fig 4)

Another type of cell to cell relation was also found it is illustrated in Figs 2 and 5–6 In the survey picture (Fig 2) one cell fulfils the

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Figs 3–4

Fig 3 Detail of Fig 1 demonstrating the type of contact usually found between the Rous mouse tumour cell and the chicken fibroblast *in the tissue culture* The two cells are clearly separated from each other The intercellular space measures about 0 5 μ Section thickness was working at 500 A × 17 000

Fig 4 Detail of close contact between microvilli of Rous mouse tumour cell and normal chicken fibroblast (bottom) A small space is visible between the intact outer cell membranes Section thickness was working at 500 A × 58 000



4

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Figs 3–4

Fig 3 Detail of Fig 1 demonstrating the type of contact usually found between the Rous mouse tumour cell and the chicken fibroblast in the tissue culture The two cells are clearly separated from each other The intercellular space measures about 0 5 μ Section thickness was working at 500 Å × 12 000

Fig 4 Detail of close contact between microvilli of Rous mouse tumour cell and normal chicken fibroblast (bottom) A small space is visible between the intact outer cell membranes Section thickness was working at 500 Å × 58 000

criteria of a chicken fibroblast with its elongated nucleus and well developed endoplasmatic reticulum Of the other cell only a small part of the nucleus is shown but the cytoplasm has the characteristic appearance of a mouse sarcoma cell with many irregular vesicles and no endoplasmatic reticulum In further sections of this cell the nucleus has a more rounded shape like that of a Rous tumour cell seen in Fig 1 Figs 5–6 demonstrate the contact between these two cells at higher magnification It is obvious that the contact between the two cells is very intimate The two adjacent cell membranes seem to be partially fused Between small vesicle like remnants of the outer membranes bridges of cytoplasmatic material are forming connections at irregular intervals Even though it is possible to follow each cell membrane for only a short distance there is no difficulty in identifying the contact between the two cells as the cytoplasm looks quite different on each side even at high magnification It is evident that the intimate contact is formed over rather large areas of the two cell surfaces as this contact could be demonstrated in at least 10 serial sections

Under unfavourable conditions oblique sectioning of a cell membrane might cause a blurring of its normally distinct outline It seems however unlikely that two adjacent plasma membranes from different cells could be sectioned obliquely in the same section length and also in several serial sections producing an identical blurring It can therefore be assumed that a very intimate contact exists between the two heterologous cells

The culture medium of mixed cultures prepared in parallel but kept continuously in fluid medium contained on the average 600 FFU/ml RSV after 12 days of co cultivation when tested according to *Temin & Rubin* (1958) Agar covered mixed cultures prepared in parellel showed apparent foci of transformation of CFF after staining with Giemsa

DISCUSSION

The initiation of virus production in mixed cultures of virus induced tumour cells and sensitive indicator cells is now a wellknown phenomenon It must be assumed to have its basis in an interaction between the two types of cells involving either a transfer of at least part

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Figs 5–6

Fig 5 Detail of intimate cell contact Chicken fibroblast (to the right) with coarse endoplasmatic reticulum Discontinuity and blurring of the cell membranes at irregular intervals along the zone of close contact. Second serial section from the survey picture Fig 2 Section thickness was working at 400 Å × 19 800

Fig 6 Intimate cell contact between the chicken fibroblast (to the right) and the Rous mouse tumour cell Note the partial fusion of the cell membrane with blurring of the two cell borders Distance from survey picture (Fig 2) about 1 μ Section thickness was working at 400 Å × 19 800

of the virus genome from the tumour cell to the indicator cell or conversely a transmission of metabolic tools absent from the tumour cell and necessary for virus synthesis. In the Rous mouse system good evidence supports the first alternative. Thus RSV production can only be demonstrated in mixed cultures of mouse sarcoma cells and intact CEF but not in cultures containing radiation damaged or sonicated CEF from which a hypothetical factor could be expected to be transferred to the mouse tumour cells (*Jonsson* unpublished). Several attempts to induce RSV synthesis in mammalian Rous tumours with different types of helper viruses have given negative results. It thus seems highly probable that the maturation of the virus particles is fulfilled in the chicken cells of the mixed cultures.

The induction of RSV synthesis in mixed cultures requires a large number of Rous mouse tumour cells (5×10–2.5×10), which is fairly constant for different tumours and different transplant passages of the same tumour (*Jonsson* 1966). These findings together with the late appearance of extracellular virus in the mixed cultures suggest either that only a small proportion of the tumour cells are carrying the viral genetic information or that the interaction between the two cell types occurs at a low frequency. Clonal analysis of a Rous rat tumour cell population (*Šimkovic et al.* 1963) gives no evidence of a segregation of tumour cells lacking viral genetic information. It thus seems more probable that the virus production is dependent upon a rather seldom occurring contact between the two types of cells. In the present material about 100 heterologous cell to cell contacts selected under the light microscope have been examined ultrastructurally. In only one of these intimate contacts cytoplasmatic bridges were observed which might offer a morphological basis for a direct transfer of high molecular substances between the two cells. On the other hand it cannot be excluded that the observed cell to cell contact via microvilli might also permit such a transfer.

The accelerating effect of Sendai virus on the production of virus in mixed cultures demonstrated both in the SV40 system (*Gerber* 1966) and in the Rous system (*Svoboda et al.* 1967) agrees with the effect of Sendai virus that is known to cause cell agglutination and fusion of cells. Also in heterologous systems Sendai virus causes a fusion and cell hybridization with an ultrastructural appearance reminding of that seen in this work (*Okada* 1962, *Harris & Watkins* 1965).

SUMMARY

Mixed cultures of Rous mouse tumour cells and chicken embryonic fibroblasts were studied in the electron microscope at the time of virus production. Usually the two types of cells are separated by a small space between the outer cell membranes. Sometimes microvilli from one cell reach the outer cell membrane of the other. Another type of

more intimate contact between a mouse tumour cell and chicken fibro blast was also observed This contact appeared as a partial fusion of the two cells with disappearance of the outer cell membranes and the for mation of cytoplasmatic bridges between the cells The possible signifi cance of these morphological types of cell to cell contacts for the ex change of genetic information is discussed

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HOST ANTIGEN IN INFLUENZA VIRUS FROM INTACT EGGS AND MEMBRANE EXPLANTS

By

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Received 29 xi 67

The haemagglutinin of influenza virus grown in the allantoic sac of the embryonated hen's egg is associated with a host antigen which has been shown to be a sulphated mucopolysaccharide (1, 4, 6, 8). In the present investigation it was examined whether this antigen is found in influenza virus from amniotic fluid, embryonic lungs and membranes. Virus grown in membrane explants was also examined.

MATERIALS AND METHODS

Viruses. The A_0/PR 8 and B/Lee virus strains were used. The PR 8 virus was inoculated into 11 days old White Leghorn eggs and incubated at 37° C for 2 days. The B/Lee virus was inoculated into 10 days old eggs, incubated at 35° C and harvested 3 days later. The virus employed in the haemagglutination inhibition (HI) test was purified by means of absorption to and elution from fowl red cells.

Anti-sera. Immune sera against host antigen were prepared in the following way: Rabbits were given three weekly intramuscular injections of 0.25 mg of purified host antigen (4) in Freund's complete adjuvant (Difco). They were bled by cardiac puncture one week after the last injection. Antisera against amniotic material were made by intravenous injections of amniotic fluid, 1 ml twice a week for 3 weeks. The rabbits were bled one week later. Non-specific serum inhibitors were removed by treatment with periodate: one volume of serum was mixed with two volumes of a freshly prepared solution of M/100 KIO_4 in distilled water. The mixture was kept over night at 37° C; finally two volumes of 5 per cent glucose were added at room temperature. The sera were then absorbed with 10 per cent fowl red cells at 4° C.

Haemagglutination inhibition test. The HI test was performed in "Perspex" trays according to the method of *Isaacs et al.* (7).

EXPERIMENTS AND RESULTS

It was important to perform the experiments in the way that virus from one source was free from contamination of virus from other sources. Before inoculation of the amniotic sac the egg was candled and the area close to the embryo marked. A square of the shell mem

brane was removed in this area the amniotic membrane lifted up and the sac inoculated using a fine needle Care was taken not to infect the allantoic sac at the same time but this could not always be prevented presumably due to leakage from the hole in the amniotic membrane The allantoic fluid was removed and the allantoic sac washed with phosphate buffered saline (PBS) before collecting the amniotic fluid

Virus from a chorio allantoic membrane (CAM) homogenate was prepared by injecting seed virus into the allantoic cavity After incubation the CAM was removed and washed several times in PBS The membrane was then ground in a mortar cell debris removed by centrifugation and finally virus in the supernatant was purified by red cell absorption elution Virus from an amniotic membrane homogenate was prepared by injecting seed virus into the amniotic cavity and treat the membrane in the same way as the CAM Care was taken not to include any of the CAM in the amniotic membrane preparation Virus from embryonic lungs was prepared by injecting seed virus into the amniotic cavity After incubation the lungs were removed and treated in the same way as the membranes

Explants were made in the following way CAMs were removed from 12 days old eggs Each membrane was cut into 5–6 pieces and each fragment placed in a test tube The fragments were washed overnight with PBS at 37° C in a roller drum The following day the membranes were washed 3 times in PBS and inoculated with PR 8 infected allantoic fluid diluted 10^{-3} in the medium and incubated in the roller drum The medium consisted of Earle s balanced salt solution to which was added 0 3 per cent lactalbuminhydrolysate penicillin 100 I U /ml and streptomycin 100 micrograms/ml In most experiments the virus was passed one or two times to new explants before being used in the HI test Virus eluates were made from the medium Explants of amniotic membranes were prepared in the same way except that each membrane was used undivided or cut into two fragments only Care was taken not to include any CAM

The PR 8 virus harvested from the different sources and purified by red cell absorption elution was examined in the HI test using rabbit antiserum against purified allantoic antigen The results are summarized in Table 1 As shown earlier virus grown in the allantoic sac was inhibited to a high titre On the other hand virus from the amniotic fluid was not inhibited Occasionally slight inhibition was observed in the stronger serum dilutions (1/5 1/10) with virus from the amniotic fluid Virus from CAM homogenates and amniotic membrane homogenates was inhibited titres varying somewhat from experiment to experiment and with different rabbit sera The titres in the table indicate the highest and lowest titres found in a series of experiments Virus from embryonic lung homogenate was not inhibited Membrane explants both CAM and amniotic gave virus which was inhibited

Results with Lee eluates agreed with those obtained with PR 8 How

ever the haemagglutinating titres of the Lee virus from CAM homogenate and membrane explants were so low that an HI test could not be performed

Results obtained with virus from allantoic and amniotic fluids tested with antiserum against amniotic material are not shown in Table 1. Allantoically grown virus was always inhibited; on the contrary, virus from the amniotic fluid was not inhibited with this antiserum

TABLE 1

Haemagglutination Inhibition Titres of Influenza Virus Harvested from Different Sources Purified and Tested with Rabbit Serum Against the Mucopolysaccharide Antigen from Allantoic Fluid

Virus from	HI titre A_0/PR 8 Pre immune serum	HI titre A_0/PR 8 Immune serum	HI titre B/Lee Pre immune serum	HI titre B/Lee Immune serum
Allantoic fluid	<5	320–640	<5	320–640
Amniotic fluid	<5	$\leqq 10$	<5	$\leqq 10$
Chorio allantoic membrane homogenate	<5	160–240		
Amniotic membrane homogenate	<5	40–640	<5	40–640
Embryonic lung homogenate	<5	<5	<5	<5
Chorio allantoic membrane explant	<5	40–320		
Amniotic membrane explant	<5	20–160		

Addition of purified allantoic mucopolysaccharide to the serum prior to the HI test blocked the inhibition showing that the inhibition was due to antibody against the normal allantoic antigen

DISCUSSION

Previous investigations have shown that the mucopolysaccharide antigen associated with the influenza virus haemagglutinin is mainly found in the allantoic and amniotic fluid, the CAM and amniotic membrane, the liver and bile and the intestinal content. The antigen has not been found in the lung (2, 5). It is assumed that the virus incorporates the antigen from the cell membrane (3). From these data it was anticipated that virus from different sources within the embryo would differ in the content of this antigen and this was found to be true

We regard the HI test as a sensitive method by which to detect the presence of the allantoic antigen on the virus particles. The fact that the haemagglutination of fowl red cells by allantoically grown influenza virus is inhibited by antiserum against the purified allantoic antigen most probably means that the antibodies against this antigen cover the virus haemagglutinin. This can be visualized if the allantoic antigen is assumed to be attached to the haemagglutinin or situated close to it. That this antigen is part of the virus has been shown definitely by isolation and characterization of the antigen from purified

virus preparation (8) By ether disruption the antigen has been shown to be associated with the haemagglutinin fraction (1)

The allantoic antigen has not been found in influenza virus passed in embryonic chick kidney monolayers (9) Our experiments showed that virus grown in the amniotic membrane contained the allantoic antigen while virus from the amniotic fluid or chick embryonic lung homogenate did not This observation indicates that most of the virus found in the amniotic fluid has multiplied in the respiratory system of the chick and not in the amniotic membrane

SUMMARY

By means of the HI test the haemagglutinin of influenza virus purified from allantoid fluid chorio allantoic and amniotic membrane homogenates and chorio allantoic and amniotic membrane explants was shown to contain the allantoic mucopolysaccharide antigen On the other hand the haemagglutinin of purified virus from amniotic fluid and embryonic lung failed to reveal the antigen This indicates that most of the virus found in the amniotic fluid has multiplied in the respiratory system of the embryo and not in the amniotic membrane

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SEROTYPING OF *PSEUDOMONAS AERUGINOSA*

1 *Studies on the Production of* Anti O Sera

By

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Recei ed 24 xi 67

Typing systems for *Pseudomonas aeruginosa* based on type specific antigens have been proposed Although some notable progress towards perfection of such systems has been attained in recent years a simple reproducible system which satisfactorily meets the requirements of the epidemiologists does not yet exist

The best results have been obtained by using heat treated cultures both as vaccines and as agglutinating suspensions The reactive antigen in this system is designated the O antigen and the antisera containing the O antibodies are called O sera Several workers (2 4 5 6 7 8 10 11) have published O antigen schemes and have examined the fre quency of the different O types in various materials of *Ps aeruginosa* isolates

The present study was planned to extend the work already done on serotyping and started with an attempt to reproduce the results of *Habs* (2) An immediate difficulty turned out to be the production of sufficiently high titered and specific O sera and so studies on the best way to produce satisfactory O sera became pertinent

MATERIAL AND METHODS

In this section a description is given of procedures comm n to all exper ments The procedures pertinent to the variation introduced in the individual experiments are described in connection with each experiment

Strains

The 12 O group strains of *Habs* were u ed ( ) The a here lesignated PJ 101 to 110 and PJ 118 and PJ 119 corresponding to O g oup 1 t 10 and O groups 11 and 12 re pectively The strains were received from *J ssen* I J c ll ction of pseudomonads (3) Their d agnosis as *Ps a ruginosa* wa c nf m d b y *J sen* The strains were kept in the lyophilized state and a fresh amp ul wa u ed whenever new batches of vaccine or antigen had to be prepared

Preparation of Antigens

Before inoculation into the fluid med m th strains were always subcultured twice on infusion agar plates at 35 C f 18 1 hours The inoculum consi t f at least 10 individual colonies from the econd plate Differences in colony ap r nce were disregarded except that a cert n type of small excavated and d lonies

was avoided The inoculum was transferred to 100 ml of infusion peptone broth in a 500 ml flask and incubated at 35 C for 18 21 hours during constant shaking The shaker moved the flask forth and back in a horizontal plane 70–80 times per minute Heating at 100 C was performed in a steamsterilizer and heating at 80 C in a waterbath The pH was measured electrometrically The density of the fluid cultures was determined by a very simple kind of nephelometry by using as a standard that density of a culture in a tube measuring 20 mm in diameter through which it is just possible to read small letters by the naked eye A culture having standard density contains about 1 5 × 10⁹ bacteria per ml Formol was added as a 20 per cent formaldehyde solution in 0 0375 M phosphate buffer to give a final concentration of 0 8 per cent formol The formol was allowed to act on the cells for 20 hours at 35 C. Washing of the cells was performed in saline (0 85 per cent sodium chloride) Coarse particles were removed from the antigen suspension by sedimentation at 5 C for 20 hours in tubes measuring 20 × 190 mm

For the sake of shortness three of the most frequently used vaccine preparations were called Vaccines A B and C These vaccines are prepared as follows

Vaccine A The vaccine used by *Habs* Twenty hour infusion peptone broth cultures heated for two hours and a half at 100 C No washing but dilution to standard density

Vaccine B An experimental vaccine developed during the study Cultures were grown in infusion peptone broth buffered by the addition of 0 03 M phosphate buffer Prior to heat treatment the cells were washed formol treated and washed again after heat treatment—at 100 C for two hours and a half—the cells were again washed and then formol treated Diluted to standard density

Vaccine C The vaccine used by *Verder & Evans* Cultures were grown overnight in infusion peptone broth heated at 100 C for two hours and a half and washed twice The vaccine had the density of the full grown culture which is about 6 to 7 times standard density

In this study shaken cultures were used in all instances whereas neither *Habs* nor *Verder & Evans* mention that their cultures were shaken

Preparation of Adjuvant Containing Vaccines

Two different adjuvants were tried *Freund*s incomplete adjuvant (1) and a calcium EDTA alginate adjuvant

To prepare *Freund*s incomplete adjuvant 85 per cent Bayol F and 15 per cent Arlacel A were mixed in an Ultra Turrax Rotor One part of the antigen suspension was emulgated manually in one part of the adjuvant and the stability of the emulsion was controlled by the drop test

The components of the alginate adjuvant were mixed according to instructions obtained from the manufacturing firm (Colab Laboratories Chicago Heights Illinois USA 1961) and antigen and adjuvant were mixed in the proportion of one part of antigen to three parts of adjuvant

The density of the antigen suspensions was adjusted according to need before the addition of adjuvant

Immunization Technique

The animals used were white rabbits bred at the Institute s own animal farm Usually only males weighing from 2500 g to 3000 g were used but in one experiment female rabbits were included Sera obtained prior to immunization never showed homologous O antibodies in dilution 1 5 The vaccines were always freshly prepared and had been controlled before use as to stability in saline and in normal rabbit serum

Details concerning vaccine preparation immunization scheme and time of bleeding are recorded separately for each experiment in the section on results

Final bleedings were performed by heart puncture under ether anaesthesia

Preservation of Sera

The blood was allowed to clot first for a couple of hours at 35 C and subsequently at 5 C for one or two days The serum portions successively separated by centrifugation were pooled leaving out the haemolysed portions and if found satisfactory kept in the frozen state (—20 C) without addition of preservatives Each animal

yielded 20–40 ml of serum which was divided into smaller lots (9 ml) to minimize the risk of secondary infection

Technique in Agglutination Test

The O agglutinations were performed in tubes measuring 10 × 70 mm containing 0 2 ml of serum dilution and 0 3 ml of antigen suspension The mixture was incubated in a waterbath at 52 C and readings were made after 4 hours and again after about 20 hours The two fold serial dilutions of the serum ranged from 1 25 through 1 6400 The antigen suspensions had a density corresponding to one half of standard density This density gave more clear cut readings than suspensions having standard density but the dilution did not change the endpoint The endpoint was determined as the last tube in the series in which a heavy precipitate occurred whereas it was not required that the supernatant should be absolutely clear In some experiments non heated formol treated antigen suspensions were used in which cases incubation of the tubes took place at 37 C Shaking of the tubes during incubation was tried in order to enhance and speed up the precipitation however no real advantage was obtained

Requirements of Antibody Titre and Specificity

For the purpose of the present study it was required that a serum should have a specific titre of not less than 800 to be accepted as satisfactory It was further required that the specificity of a serum should be so that its heterologous reactions with the other group strains did not exceed the values reported by *Habs*

RESULTS

Altogether nine different immunization experiments were performed Some of them were run simultaneously but in most cases the results of the already finished experiments determined the nature of the subsequent experiment To facilitate the discussion the experiments are numbered from 1 through 9 in chronological order

Experiment 1

Heat treated Vaccine Prepared According to Habs *Method Given Intravenously According to* Pelczar s *Immunization Scheme*

Vaccine 12 strains Vaccine A standard density

Animals 3 male rabbits per strain

Course Three weekly series each consisting of 3 injections on three consecutive days Amount in ml of vaccine injected (0 2 + 0 4 + 0 4) + (1 0 + 2 0 + 2 0) + (2 0 + 2 0 + 2 0)

Time of bleeding Seven days after the last injection

Results Two strains (PJ 105 PJ 118) evoked titres of 200 in 4 rabbits In all the remaining animals antibodies were present at very low titres (25 or 50) or absent

Comment This very unsatisfactory result was at first interpreted as being due to an ineffective vaccine and therefore led to experimentation on methods of vaccine preparation and the development of the experimental Vaccine B

Appendix to Experiment 1

At first it was assumed that the poor sera were due to an unsatis factory vaccine and therefore different ways of preparing the vaccine were tried The variations examined included different degrees of heat treatment a stronger buffering of the growth medium addition of formaldehyde before heat treatment and finally combination of the two latter procedures supplemented by washing of the suspensions at different stages The agglutinability of the suspensions was first tested and later some of them were tried as vaccine The outcome of these ex periments was not very encouraging but in retrospect it was realized that the poor results were probably due to the fact that the density of the vaccines was not optimal and that the bleeding of the animals was performed too late

A few useful observations were made however (1) Heat treatment at 80 and 100 C for periods varying from 30 minutes to 3 hours was found to be equally effective (2) Formaldehyde added before heat treatment improved the agglutinability considerably (3) Growth in a strongly buffered medium also seemed to enhance agglutinability some what but a combination of buffered medium and formaldehyde treat ment may result in unstable suspensions if the buffer and the form aldehyde are not removed by washing before heat treatment takes place The interpretation of these latter results is not quite clear Pos sibly the formaldehyde treatment has the effect of inhibiting the libera tion of the antigen molecules from the cell during the heat treatment and as it seems that this protective effect is more pronounced in an acid environment than in a neutral one it seems likely that heating in an acid environment increases the liberation of O antigen molecules (4) Finally observations were made which suggested that the orga nism besides the O antigen may possess another heat stable surface antigen capable of inhibiting the reactivity of the O antigen

Experiment 2

Experimental Vaccine B Given Intravenously According to Pelczar's *Immunization Scheme*

Vaccine All 12 group strains experimental Vaccine B standard density

Animals 3 male rabbits per strain

Course As in Experiment 1

Time of bleeding Nine days after the last injection

Results In 3 animals the titres reached 800 the remaining showed homologous titres ranging from 25 to 400 with a majority having titres from 50 to 100

Comments Although somewhat better results were obtained the sera were still far from being satisfactory As the preparation of ex perimental Vaccine B was very time consuming and its efficacy not

very convincing its use was discontinued and it was decided to try the immunization procedure described by *Verder & Evans* in 1961 (8)

Experiment 3

Vaccine Preparation and Immunization Course According to Verder & Evans

Vaccine One strain only PJ 103 Vaccine C 7 times standard density

Animals 3 male rabbits

Course A total of 5 injections given intravenously at intervals of four days Amount of vaccine in ml at each injection 0 25 0 50 1 0 1 5 2 0

Time of bleeding Trial bleedings at 4 7 9 and 11 days after the last injection

Results At the first trial bleeding 2 animals showed antibody titres of 800 and 1 animal a titre of 400 At the next bleeding the titres had in all cases dropped one step in the dilution series and at this level they remained during the following bleedings

Comments The results suggest that a high vaccine density and an early bleeding are of importance Before this lead was followed it was decided to try the effect of adding adjuvants to the vaccine

Experiment 4

Vaccine C and Adjuvant Vaccines Given Subcutaneously According to the Immunization Scheme of Verder & Evans

Vaccines One strain PJ 103 Comparison of three different vaccines (1) Vaccine C (2) Vaccine C + calcium EDTA alginate adjuvant (3) Vaccine C + *Freund*s incomplete adjuvant 7 times standard density

Animals Each vaccine given to 10 male rabbits

Course As in Experiment 3 except that 2 of the animals receiving Vaccine C + *Freund*s adjuvant were only given one injection

Time of bleeding In all animals trial samples were taken 4 7 11 15 19 26 33 40 47 and 54 days after onset of vaccination In 4 animals samples were also taken 62 68 84 and 92 days after onset

Results Animals given Vaccine C and Vaccine C + calcium EDTA alginate adjuvant subcutaneously did not produce any serum with a homologous titre above 25 and in a majority of the animals antibodies were not demonstrable at any time during the experiment

The results obtained with Vaccine C + *Freund*s incomplete adjuvant are shown in Table 1 The results were fairly satisfactory as far as titre height goes Three animals out of the 8 receiving a full course of immunization produced sera with titres of 800 or above in 3 animals a titre of 400 was obtained and in the remaining 2 the titre did not exceed 200 In general the highest titre was not obtained until 5 to 6

weeks after the beginning of the course Once the maximum titre of the animal was obtained it showed little tendency to decline within a period of 3 months as shown for the 4 animals observed for that period

TABLE 1 (Experiment 4)

Homologous O Agglutinin Production Response of Rabbits to Subcutaneous Injection of Adjuvant Vaccine

Strain	Animal No	O titre														
	806		0	0	0	0	0	0	25	50	50	100	50	50	50	25
	807		0	0	0	0	0	50	50	200	200	400				
	808		0	0	0	0	0	25	100	100	100	00	200	200	200	200
	809		0	0	0	0	100	400	800	[illegible]	800	800				
PJ 103	810		0	0	0	0	50	200	800	800	400	400				
	811		0	0	0	0	50	50	200	200	200	200				
	812		0	0	0	25	50	200	400	800	800	800				
	813		0	0	0	25	25	50	200	400	200	400	400	400	200	200
	814		0	0	0	25	50	100	200	400	200	100				
	815		0	0	0	0	50	200	200	200	200	200	200	400	200	200
Vaccine injection		↑	↑	↑	↑	↑										
Day of trial bleedings§		0	4	7	11	15	19	26	33	40	47	54	62	68	84	92

One injection only was given § Days after first injection

The heterologous reactions of the sera were not determined

Comments When compared with Experiment 3 the results with plain Vaccine C show that the subcutaneous route of administering plain vaccine is much inferior to the intravenous route in fact subcutaneous injections produce hardly any antibody response

The equally poor results with Vaccine C + calcium EDTA alginate adjuvant show that this adjuvant apparently had no effect at all An explanation for this may be that a satisfactory emulgation was not achieved This in fact was suspected already at the outset of the experiment

In an attempt to improve the results obtained with Vaccine C + *Freund*s adjuvant it was decided to examine the effect of two intravenous booster injections given to the already subcutaneously vaccinated animals

Experiment 5

Effect of Two Intravenously Given Booster Injections of Plain Vaccine C to Animals Already Vaccinated Subcutaneously with Vaccine C + Freund s Incomplete Adjuvant

Vaccine Strain PJ 103 Vaccine C 6 5 times standard density

Animals The immunized animals Nos 807 809 810 811 812 and 814 from Experiment 4 (see Table 1)

Course Two injections of 2 5 ml of vaccine were given i v the first 6 weeks after the last subcutaneous injection of adjuvant vaccine and the second 4 weeks later

Time of bleeding Trial samples were taken 3 5 7 11 27 31 33 and 35 days after the first i v booster injection

Results The results are shown in Table 2 Five days after the first booster injection all animals showed a definite rise in antibody titre and on the 11th day 5 out of the 6 animals reached titres of 800 or above All titres declined somewhat within the next 2 weeks but went up again after the second booster injection

TABLE 2 (Experiment 5)

Homologous O Agglutinin Production Response to Intravenous Booster Injections of Vaccine C of Rabbits Previously Immunized Subcutaneously with Adjuvant Vaccine

Strain	Animal No	O titre										
	807	400§		400	1600	1600	1600	800		800	800	800
	809	800		800	1600	800	800	800		400	1600	800
PJ 103	810	400		400	800	400	800	200		200	800	200
	811	200		100	400	200	400	100		100	200	200
	812	800		1600	3200	3200	1600	1600		800	1600	1600
	814	100		400	400	800	800	200		200	800	400
Booster injection			†						†			
Day of trial bleeding†		54	57	60	62	64	68	84	85	88	90	92
Day of trial bleeding			0	3	5	7	11	27	28	31	33	35

This animal received only one subcutaneous injection of adjuvant vaccine
§ Antibody titre prior to first booster injection (see Table 1)
† Days after first subcutaneous injection
Days after first intravenous injection

Comments The results indicate that sufficiently high titered sera could be obtained by a combination of the procedures from Experiments 4 and 5 However the specificity of the sera was not satisfactory as heterologous titres up to 200 were observed with some of the other O group strains The serum from animal No 807 which received only one subcutaneous injection of adjuvant vaccine showed heterologous reactions at a somewhat lower level than the other sera indicating that the number of subcutaneous injections increases the degree of unspecificity of the sera

TABLE

Homologous and Heterologous O Agglutinin Titres Obtained by Immunization with the Different Immunization Procedures

Immunization technique	O group	Animal no	Strain 101	102	103
a	1	831	1600	100	200
b		938	1600	0	0
c		1040	12800	25	50
a	2	839	200	400	200
b					
c		943	0	800	0
e		1054	50	3200	50
a	3	844	200	50	800
b		950	25	0	1600
a	4	848	100	50	50
b		958	0	0	0
c		958	100	50	50
d		1095	0	0	0
e		1093	25	50	25
a	5	852	200	100	200
b		964	0	25	0
e		1059	25	200	25
a	6	857	400	100	200
b					
c		966	25	0	0
e		1066	100	50	100
a	7	861	200	100	200
b		973	50	50	100
a	8	870	200	100	100
b		980	0	0	0
a	9	871	200	100	100
b					
c		986	0	0	0
a	10	879	200	100	200
b		992	0	0	0
d		1106	0	0	25
e		1101	25	25	25
a	11	894	100	50	100
b		995	0	0	0
c		995	25	0	25
e		1074	100	50	100
a	12	899	200	100	200
b		1006	0	0	0

* No serum tested

a Results with sera from Exp 7 subcut adjuvant vaccine + subsequent i v booster

b Results with sera from Exp 8 i v vaccine about 7 times standard density

Experiment 6

A Course of Vaccine C Given Intraperitoneally Followed Later by One Intravenous Booster Dose

Vaccine Strain PJ 103 only Vaccine C 8 5 times standard density

3
12 O Group Strains of Habs a Comparison of the Degree of Specificity Obtained by the Dif
used in Experiments 7 8 and 9

(PJ no) 104	105	106	107	108	109	110	118	119
100	200	100	400	100	200	200	200	100
0	0	0	0	0	0	0	0	0
50	50	25	25	25	25	50	50	25
100	100	50	400	100	200	100	200	100
0	0	0	0	0	0	0	0	0
2	200	25	2›	25	25	50	100	0
50	50	50	400	100	100	50	100	50
0	0	0	0	0	0	0	25	0
400	50	50	100	50	50	100	50	50
3200	0	0	0	0	0	0	25	0
800	50	50	100	50	50	50	100	50
3200	0	0	0	0	0	0	0	0
1600	50	25	50	25	25	25	50	25
100	1600	100	400	200	200	200	200	100
0	800	0	0	0	0	0	0	0
0	1600	0	0	0	25	25	25	0
100	100	800	800	200	200	200	200	100
0	0	800	0	0	0	0	0	0
50	100	1600	100	100	100	100	100	50
100	100	100	1600	200	200	100	100	100
50	50	50	1600	100	50	50	50	50
100	100	100	400	1600	200	100	100	100
0	0	0	100	800	0	0	0	0
100	100	100	200	100	1600	100	100	100
0	0	0	0	0	800	0	0	0
100	100	100	800	200	400	400	200	100
0	0	0	0	0	0	800	0	0
0	0	0	0	0	0	1600	0	0
0	25	50	25	50	50	1600	50	0
100	50	50	200	100	100	100	800	25
0	0	0	0	0	0	0	3200	0
0	25	25	0	25	0	[illegible]	1600	0
[illegible]	50	25	50	50	50	100	12800	25
200	100	50	400	200	200	100	[illegible]	400
0	0	0	0	0	0	0	0	800

c Results with sera from Exp 8 i v vaccine + ubsequ nt v booster ab ut 7 tim s standar l density
d Results with sera from appendix to Exp 9 i vacc n 25 times tandard density
e Results with sera from Exp 9 i v vaccine 100 times tan l rd density

Animals 5 male rabbits

Course After five i p injections of 0 25 0 50 1 0 1 5 and 2 0 ml of vaccine given at intervals of 4 days one i v injection of 2 5 ml of vaccine was given 12 days after the last i p injection

Time of bleeding Trial samples were collected 3 5, 7 and 10 days after the last i p injection and again 5 6 7 and 8 days after the booster injection

Results Three days after the last i p injection titres ranged from 25 to 200 and in the course of the following week 4 of the 5 sera showed a small decline in titre Five days after the i v booster dose all sera showed a titre of 200 and no appreciable change during the following 3 days

Comments A comparison between this experiment and Experiment 3 indicates the advantage of the intravenous route over the intraperitoneal route The booster effect although recognizable was not sufficiently pronounced to make the procedure of the experiment recommendable

Experiment 7

A Course of Three Subcutaneous Doses of Vaccine C + Freund's Incomplete Adjuvant Followed by Two Intravenous Booster Doses of Plain Vaccine C

This experiment is based on the information obtained in Experiments 4 and 5 The number of subcutaneous injections was reduced from 5 to 3 in the hope that this would improve the specificity

Vaccines All 12 O group strains Vaccine C + *Freund's* adjuvant and plain Vaccine C 6 to 8 times standard density

Animals 5 male rabbits per strain

Course After three doses of 0 25 0 50 and 1 0 ml of adjuvant vaccine given subcutaneously at intervals of 4 days a first booster dose of 2 5 ml of plain Vaccine C was given i v 7 to 8 weeks after the last subcutaneous dose The second booster dose was given 1 week after the first one

Time of bleeding Trial samples were collected once a week in the interval between the last subcutaneous dose and the first i v one Further samples were collected 4 5 6 and 7 days after each of the booster injections

Results a) *Titre values* After the subcutaneous doses were given antibody titres remained fairly low 5 animals only showing a titre of 400 After the first booster dose 18 animals reached a titre of 800 or above and after the second booster dose an additional 11 animals reached this level At this stage 4 animals only showed titres below 200 With each strain at least one serum was obtained with a titre of 800 or higher

b) The specificity of selected representative sera is shown in Table 3 A single look at this table is enough to disclose that these sera are not sufficiently specific to be used for typing purposes In several instances there is only a difference of one or two dilution steps between the homologous and some of the heterologous titres

Comments This experiment was complicated by local infections

developing at a majority of the injection sites resulting in open necrotic ulcerations in all the animals Only 2 of the animals succumbed The ulcers were healed before the booster injections were given *Ps aeruginosa* could not be isolated from any of the ulcers whereas another pseudomonad *Ps maltophilia* was isolated from a few of them Experience in this laboratory indicates that *Ps maltophilia* is an organism possessing only a very low degree of pathogenicity to human beings and it was therefore considered unlikely that it caused the local infection in the rabbits The sera did not agglutinate antigen suspensions (one formol treated one heat treated) prepared from one of the isolated strains of *Ps maltophilia* The question of the cause of these local infections therefore remains unanswered which applies also to the question whether or not this complication contributed to the poor specificity of the sera

Experiment 8

A Course of Five Intravenous Doses of Plain Vaccine C Followed by Two Intravenous Booster Doses of the Same Vaccine about 8 Weeks Later

As the results so far obtained suggested that the procedure of *Verder & Evans* was the best one Experiment 8 is basically a repetition of Experiment 3 but supplemented by late booster doses which after the foregoing experience would appear to be a useful adjunct to the procedure The importance of the sex of the rabbits was also studied in this experiments

Vaccine All 12 group strains Vaccine C For the booster doses fresh batches of vaccine were prepared The density of the individual vaccines in both batches ranged from 4 5 to 8 5 times standard density with a majority lying between 6 0 and 7 5 times standard density

Animals 3 male and 3 female rabbits per strain

Course The initial round was exactly as in Experiment 3 The first of the two booster doses (2 0 ml) was given 8 weeks after the last dose in the initial round and the second (2 5 ml) 1 week later All doses were given intravenously

Time of bleeding As regards 3 strains (PJ 110 PJ 118 PJ 119) trial samples were collected 3 and 4 days after the fourth injection After the fifth injection trial samples were collected after 5 6 and 7 days for 6 of the strains and after 3 4 5 and 6 days for the other 6 strains

Results As an example detailed results for 3 representative strains are shown in Table 4 The results with strains PJ 103 and PJ 118 are representative for one half of the strains after the initial round while the results with strain PJ 108 are representative for the other half of the strains Quite satisfactory results were obtained with strains PJ 103 and PJ 118 8 of the 12 animals showed titre values of 800 or higher With strain PJ 108 however 1 out of 6 animals only gave high titered sera The antibody level dropped rapidly 7 to 8 weeks after the fifth

TABLE 4 (Experiment 8)

Homologous O Agglutinin Production Response of Rabbits to Intravenous Injection of Vaccine C from Selected Strains about 7 Times Standard Density and Subsequent Intravenous Booster Injections

Strain	Animal No	Sex	4th inject			5th inject						1st booster					2nd booster				
			O titre values after the indicated vaccine doses																		
PJ 103	947	♂						800	400	50	0	0	100	100	100		100	200	200	200	200
	948	♂						800	100	50	0	0	100	100	100		100	200	200	200	100
	949	♂						800	50	50	0	0	100	100	100		100		200	400	200
	950	♀						3200	1600	800	25	0	100	200	400		400	800	800		
	951	♀						100	100	200	0	25	100	50	50		100	400	800		
	952	♀						800	400	50	0	25	50	100	100		200	400	400	400	200
LJ 119	995	♂	3200	1600	800	§					0	100	200	100	400	100	400	1600	1600		
	996	♂	50	0		50	100	400	100		0	0	100	100	100	50	50	800	1600	1600	
	997	♂	50	50		50	100	100	100		0	0	50	100	100	100	50	800	400	800	200
	998	♀	1600	800	400	§					0	50	100	100	100	50	100	1600	1600		
	999	♀	1600	800	800	§					25	100	200	800	800	100	200	1600			
	1000	♀	400	200		100	200	100	100		0	50	200	100	100	100	100	3200			
LJ 108	977	♂				100	25	50	50		0	0	50	50	100	50	50	200	100	50	100
	978	♂				200	100	100	200		0	50	100	200	100	100	200	200	400	200	100
	979	♂				200	100	400	200		0	0	400	400	400	400	400	400	400		100
	980	♀				800	400	400	200		25	50	100	200	400	200	800	1600			
	981	♀				200	50	100	50		25	50	100	400	200	200	200	400	400	400	
	982	♀				200	50	50	100		0	50	100	100	100	200	100	200	200	200	100
Day of trial bleeding			3	4	7	3	4	5	6	7	53	3	4	5	6	7	3	4	5	6	7

♂ male rabbit
♀ female rabbit
Serum not tested

§ 5th injection not given
Days after injection of indicated vaccine dose

injection antibodies could practically no longer be demonstrated The booster doses produced a great number of high titered sera for 8 strains including PJ 118 an unaltered low number of high titered sera for 2 strains (PJ 108 and PJ 106) and a reduction in the number of high titered sera for 2 strains (PJ 103 and PJ 107) The table shows that the first booster produced only a moderately good response 1 out of 18 animals reached a titre of 800 The varying densities of the different vaccines are not reflected in the titre heights obtained

The specificity of a selected high titered serum for each strain is shown in Table 3 The results are from blood samples collected within the first week after the termination of the initial round No high titered sera were available for strains PJ 102 PJ 106 and PJ 109 With one exception (serum 973 representing O group 7) there are very few heterologous reactions and those occurring are at very low titres compared with the homologous ones Examination of samples collected after the booster doses had been given showed that the specificity was impaired in sera from 13 out of 24 animals (9 strains) which only gave high titered sera after booster doses In Table 3 these sera are represented by selection of a high specific serum for each of the strains PJ 102 PJ 106 and PJ 109

Furthermore 17 animals (9 strains) which gave high titered sera both after the initial round and after booster doses showed an impairement of the specificity after booster doses in sera from 5 animals In Table 3 sera for 2 strains (PJ 104 and PJ 118) have been selected which show 1–3 dilution step higher cross reactions after booster doses

Comments In this experiment sera were obtained with all 12 strains which met the initially formulated requirements both as to titre height and specificity Three practical difficulties are apparent one is the variation in the antibody response of the individual animals the second is the difference of antigenicity among the strains and the third is the rapid drop in antibody level which may occur In some cases a very high titre may in the course of a few days mysteriously be reduced to a moderate or even a low titre Repetition of the agglutination tests in such cases showed that these results were not due to titration errors

Experiment 9

Effect of Increasing the Density of Vaccine C

In general more high titered sera were obtained with Vaccine C than with Vaccine A Since Vaccine C mainly differs from Vaccine A in having a higher density Experiment 9 was performed to examine the effect of increasing the density of the vaccine

Vaccines were prepared from strain PJ 101 102 104 105 106 110 and 118 The vaccines were prepared as Vaccine C but concentrated so that the density was 100 times standard density

Animals 6 male rabbits per strain

TABLE 5 (Experiment 9)

Homologous O Agglutinin Production Response of Rabbits to Intravenous Injection of Vaccine C from selected Strains 100 Times Standard density and to Subsequent Intravenous Booster Injections

Injections →		1st	2nd	3rd			4th				5th					1st booster			2nd booster	
Strain	Animal No	O titre values after injection of indicated vaccine doses																		
PJ 101	1040				6400		6400				6400	12800	12800	6400	0	†				
	1041				3200		6400				6400	6400	3200	3200	0	25	400		1600	6400
	1042			†																
	1043				6400		6400				6400	6400	6400	3200	0	100	3200			
	1044				6400		6400				6400	6400	3200	3200	25	200	1600		6400	
	1045				6400		6400				6400	3200	3200	3200	0	50	200		1600	6400
PJ 118	1070	50	3200	6400	6400	6400	†													
	1071	50	800	12800	12800	12800	3200	1600	1600	3200	§				0	100	6400		6400	
	1072	25	6400	25600	12800	12800	3200	3200	1600	200	†									
	1073	50	1600	12800	12800	6400	1600	6400	3200	6400	§				25	800	3200		6400	12800
	1074	100	3200	12800	12800	12800	6400	6400	3200	3200	§				25	400	6400	12800		
	1075	50	1600	25600	25600	12800	6400	6400	1600	1600	§				0	800	6400		6400	25600
Day of trial bleeding		4	4	3	4	5	4	5	6	7	3	4	5	7	105	3	4	5	3	4 5

Serum not tested
§ 5th injection not given
2nd booster not given

† Death of animal
Days after injection of indicated vaccine dose

Course An initial course consisting of three (strains PJ 104 and 110) four (PJ 105 106 and 118) or five (PJ 101 and 102) i v injections was given with intervals of 4 days Two i v booster injections were given 14 weeks later with an interval of 4 days The volume of vaccine given at each injection was 4 times higher than usual because it was found necessary to make a four fold dilution of the very dense vaccine immediately before injection to escape injection of large aggregates of bacteria The volume of the successive injections in the initial course was therefore 1 0 + 2 0 + 4 0 + 6 0 + 8 0 ml and of the two booster injections 2 0 + 3 0 ml

Time of bleeding As shown in Table 5 from 1 to 5 samples were taken after each vaccine injection the first one after 3 or 4 days

Results The results obtained with 2 representative strains are shown in Table 5 It is remarkable that very high titres were obtained already 4 days after the second injection A further considerable rise followed after the third injection At this time the antibody level seemed to have reached its maximum since higher values were rarely seen later in the course Fourteen weeks after the termination of the initial course almost all antibodies had disappeared but 3 or 4 days after the injection of the two booster doses most of the animals had again reached the maximum level

The specificity of samples taken at the end of the initial course is shown in Table 3 Most sera reacted with almost all strains but the heterologous reactions were weak the titre rarely exceeded 50 except in cases where it is known from *Habs* that common partial antigens exist However most of the high titered sera obtained after the booster doses gave heterologous reactions with titres of 100 or 200 with all strains

Comments The favourable effect on the titre height of increasing the density of the vaccine was clearly demonstrated it can also be seen that this good result is obtained at the expense of some loss in specificity There is one practical draw back connected with the use of the very dense vaccine it is badly tolerated by the animals 15 per cent of the animals died 1 to 3 days after an injection and deaths in general occurred more frequently than usual All the animals appeared sick and did not eat normally

Appendix to Experiment 9

In parallel with the trial of vaccines of a density 100 times standard density two other vaccines were tried One was a very dilute one having a density of only 0 001 times standard density corresponding to about 10⁶ bacteria per ml the other had a density of 25 times standard density Both were prepared as C vaccines The procedure was as in Experiment 9 with minor deviations but a detailed description will not be given As expected the dilute vaccine provoked a rather poor re

sponse although a few animals reached the titre value of 800 after the five initial injections When the titres had declined to about zero two booster injections were given After this the titres rose again but did not reach the level obtained after the initial course

As regards the vaccine with a density of 25 times standard density only the three first doses of the initial course were given and no booster injection Titres after the three injections ranged from 1600 to 6400 and the specificity was very good as shown in Table 3

DISCUSSION

Among the many variables involved in the practice of producing high titered and specific antisera the following have received more or less attention in the present study

1 Varying degrees of buffering of the medium in which the antigen is produced
2 Formol treatment of the antigen
3 Varying degrees of heat treatment of the antigen
4 Washings of the cell suspensions at various stages during antigen production
5 *Variations in the total amount of antigen injected*
6 Addition of adjuvant to the antigen
7 Variations in the route of introduction of the antigen
8 Variations in the time relationship of the individual antigen injections especially the booster effect
9 Variations in the duration of the total immunization period
10 Variations in the time of bleeding in relation to the immunization course
11 The sex of the immunized animals
12 Variation in the antibody producing capacity of the individual animals

From the outset the study was not planned as a systematic examination of all the factors just mentioned The experiments were allowed to develop one from the other with more emphasis being laid on the ultimate goal that of obtaining sufficiently good sera than on an experimentation which afterwards would allow definite conclusions to be drawn concerning the influence of the variables introduced

As regards some of the items in the above list further particulars are not required to what has already been said either because the information is far too incomplete to warrant further discussion (Items 1 2 4) or because the results seem to be very definite (Items 3 11)

Among the informations obtained the following should be especially stressed from the point of view of obtaining good sera a *large*

amount of antigens should be introduced (Item 5) by the *intravenous* route (Item 7) the immunization course should be of *short* duration (Item 9) and the bleeding should follow only a *few days* after the last injection (Item 10)

The very large amount of antigens used in Experiment 9 is probably above requirements As far as the information goes (Appendix to Experiment 9) it seems that a density of about 5×10^{10} bacteria per ml corresponding roughly to a five fold concentration of a full grown culture will be sufficient This conclusion refers to an antigen treated according to *Verder & Evans* (8) A vaccine of this more moderate density did not harm the animals as the very concentrated vaccines did even after some dilution before injection

That the intravenous route is clearly superior both to the subcutaneous and the intraperitoneal route can be concluded from a comparison of Experiments 4 6 and 8

The importance of using only a short immunization period to obtain highly specific antibodies has often been pointed out in the literature It has been amply confirmed in this study as seen especially in Experiment 8 In Experiment 9 in which the very dense vaccine was used the specificity of comparable sera was not quite as good as in Experiment 8 The reason for this is not known but it is tempting to relate the reduced specificity and the high density of the vaccine Anyway also this observation suggests the use of a moderately dense vaccine instead of a very dense one

The optimal time for bleeding of an immunized animal from the point of view of obtaining both high titered and specific sera is often difficult to determine because the period during which specific antibodies are at their highest level may be very short and may vary from one animal to another It is apparent from the experiments that this difficulty is reduced when dense vaccines are used partly because it looks as if a high specific level is maintained for a longer period when large amounts of antigen have been introduced and partly because it looks as if the variations among the animals become relatively less important when the general antibody level is high If the experience gained in Experiment 9 is followed it looks as if the optimal time for bleeding would be 3 or 4 days after the third intravenous injection

The kind of immunization scheme followed during the initial course does not seem to be of paramount importance The scheme of *Verder & Evans* i e single injections given at intervals of 4 days is convenient and may be recommended as shown in several of the experiments The use of booster doses many weeks after an initial course is clearly not advisable because of the resulting reduced specificity Therefore it does not seem to be a rational procedure to keep the animals after the first bleeding and to continue immunization if the need for greater quantities of sera arises If a large consumption of serum is expected it is more economic to start with a sufficiently large number of animals and

The protein concentration was calculated from light absorption at 280 nm ($E_{1\,cm}^{1\,per\,cent} = 12.7$) in phosphate NaCl buffer

Separation of heavy (H) and light (L) chains A 1 per cent solution of IgG in 0.01 M Tris pH 8.0 was reduced in 0.1 M mercaptoethanol and subsequently alkylated in 0.2 M iodoacetamide according to *Fleischman et al* (1963) made 0.03 M in sodium dodecylsulphate (SDS) (kindly supplied in a purified form by Lilje holmens Stearinfabrik Stockholm) and dialysed over night against 0.03 M SDS in 0.01 M Tris at pH 8.0 essentially in the way described by *Karush & Utsumi* (1964) Separation of H and L chains was performed on a column (2.5 × 45 cm) of Sephadex G 200 equilibrated with 0.03 M SDS in 0.01 M Tris pH 8.0 and 0.002 M EDTA The effluent was collected with a fraction collector in 4.5–5 ml fractions and passed through an UVI cord (LKB Stockholm) analyser operating at 2537 A to locate the protein peaks

Agar gel electrophoresis was run in 1 per cent agarose in diemal buffer at pH 8.6

Removal of sodium dodecyl sulphate from protein solutions —Method 1 The protein solutions containing detergent were mixed with about 2/5 of its total volume of packed Dowex 1 acetate (*Jaquet et al* 1964) The Dowex 1 acetate was kept suspended for 10 minutes and removed by centrifugation

Method 2 The protein solution containing detergent was dialysed for 2 days against Tris buffer containing Dowex 1 acetate (*Karush & Utsumi* 1964) Residual detergent was estimated by the ability of the solution to lyse red blood cells minimum concentration detectable 10 M

Sedimentation analysis The sedimentation analysis was done in a Spinco Model E analytical ultracentrifuge at 59 780 r p m at 20 C The s values were determined in the way previously described (*Hansson et al* 1966) Reduced IgG was centrifuged in the presence of SDS The sedimentation values determined in this buffer system were given as s_{201} at 20 C. The sedimentation values determined in other buffer systems were converted to s values in water at 20 C $s_{20\,w}$

Adenovirus neutralization tests In the tests the rapid plaque forming variant type 5 r (*Kjellen* 1963) was used throughout

Standard test Equal volumes of virus (10–10 PFU/ml) and IgG or fractions of it were mixed The mixtures were incubated at room temperature for 2 hours After dilution in phosphate buffered saline (PBS) pH 7.4 the mixtures were seeded onto monolayers of MAS cells (*Kjellen* 1961) and assayed for surviving PFU On the 5th day after inoculation the plates usually 3 4 per dilution step were overlaid with a second agar medium containing neutral red PFU were counted on the 7th–10th day

Enhancement test The technique was used as an indicator of a combination between virus and homologous antibody To a virus rabbit antibody mixture pre incubated for 2 hours at room temperature a goat anti rabbit H chain or L chain serum was added (*Goodman & Donch* 1965) The fractions of surviving virus were assayed on monolayers after additional incubation for 2 hours at room temperature

Low pH test Equal volumes of virus suspended in Tris HCl buffer at pH 3.0 and of the fraction dissolved in glycine HCl buffer at pH 3.0 were mixed After incubation for 1 hour the mixture was dialysed for 2 hours against Tris buffer pH 8.0 before assayed according to the standard test

RESULTS

Analysis of the IgG and the Separated Fractions

On ultracentrifugal analysis the IgG showed one single symmetrical peak with an s value ($s_{20\,w}$) of 6.70 (more than 97 per cent of the molecules within s value close to 7.0) On electrophoresis in agar gel the IgG preparation revealed no inhomogeneity that could not be explained by the heterogeneity of the IgG itself (more than 90 per cent of the 7 S molecules are IgG)

After fractionation of the reduced and alkylated IgG on Sephadex

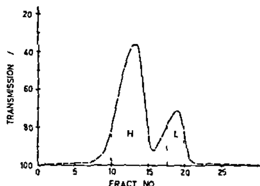


Fig 1

Elution pattern of reduced alkylated and SDS treated rabbit IgG Column (2 5 × 45 cm) of Sephadex G 200 equilibrated with 0 03 M SDS and 0 002 M EDTA in Tris buffer 0 01 M pH 8 0 Volume of fractions 4 5 ml Marked areas indicate effluent pooled to yield H chain and L chain fractions

G 200 in the presence of SDS 2 peaks were obtained (Fig 1) The first peak the H chain fractions contained about 2/3 of the original protein amount For further analysis this peak was divided into three parts A was a pool of tubes 11 and 12 B was a pool of tubes 13 and 14 and C was tube 15 The second peak the L chain fraction was represented by tubes 19 and 20

On analysis in the ultracentrifuge fraction A showed a single peak with an s value (s_{0b}) of 3 0 Fraction B and C showed single peaks with s values (s_{20b}) of 2 7 and the L chain fraction showed one peak with s value (s_{20b}) of 2 5 under the same conditions (Fig 2)

Native IgG dissolved in SDS tris buffer and kept in solution in this buffer for the same lenght of time as that used for the fractionation procedures of the reduced an alkylated IgG showed an s value (s_{20l}) of 4 0

When dissolved in SDS native untreated as well as the reduced and alkylated specimen showed a high anodal electrophoretic mobility on agarose gel electrophoresis This was in contrast to the more cathodal mobility shown when the IgG was dissolved in buffers free of SDS (Fig 3) No significant difference in electrophoretic mobility could be observed between fractions A B C and the L chains in the presence of SDS

None of the H chain fractions had any appreciable amounts of protein in solution after removal of SDS in accordance with method 1 Fraction A B and C precipitated when SDS was removed by method 2 and only 1 per cent of the original protein remained in solution The L chain fraction remained clear after removal of SDS (method 2) and the recovery of proteins was 100 per cent

The insoluble precipitates of fractions A B and C after removal of SDS remained insoluble in Tris buffer at pH 3 0 The fractions were

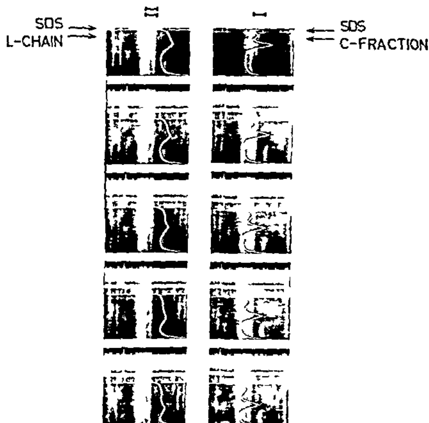


Fig 2

Sedimentation patterns of fractions from SDS dissociated reduced and alkylated rabbit IgG after filtration on Sephadex G 200 —I Fraction C dissolved in Tris buffer pH 8 0 containing 0 03 M SDS Speed 50740 rpm First picture was taken 108 min after reaching indicated speed Consecutive pictures at intervals of 16 min —II L chain fraction dissolved in Tris buffer containing 0 03 M SDS Speed 50740 rpm First picture taken after 168 min Consecutive pictures at intervals of 16 min

however rendered soluble in glycine HCl buffer at pH 3 0 After dialysis of the glycine HCl solutions against Tris buffer at pH 3 0 the fractions remained in solution After dialysis of the glycine HCl solutions against Tris buffer at pH 8 0 the solutions were slightly opalescent

Neutralization tests

The untreated IgG preparation The results of standard and enhancement tests with varying concentrations of untreated IgG are given in Table 1 In all the concentrations tested the IgG had a neutralizing effect on virus An addition of goat anti rabbit H chain or L chain serum to the mixtures of virus and IgG produced an additional significant reduction in virus survival At the dilution used 1/100 neither the goat anti rabbit H chain nor the anti rabbit L chain serum had any neutralizing effect on virus

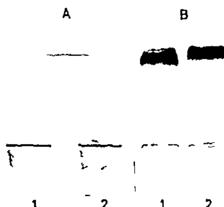


Fig 3

Agar gel electrophoretic pattern of rabbit IgG
A Preparations dissolved in Tris buffer 0 01 M pH 8 0 Electrophoresis for 1 hour
1 IgG 2 Reduced and alkylated IgG
B Preparations dissolved in Tris buffer 0 01 M pH 8 0 containing 0 03 M SDS
Electrophoresis for 15 min 1 IgG 2 Reduced and alkylated IgG

TABLE 1
Neutralizing Capacity of IgG

Original mixture		Fraction of virus escaping inactivation after addition of		
IgG mg/100 ml	Adenovirus PFU/ml	buffer	goat anti H	goat anti L
5	1 8 × 10	6 6 × 10	4 8 × 10⁻⁴	5 8 × 10
10	1 ~ × 10	3 0 × 10	9 ? × 10	9 1 × 10
30	3 0 × 10	1 6 × 10	0	n t §

No plaques at dilution 1/10
§ n t = not tested

The soluble parts of fractions A B and C As mentioned above 1 per cent only of fractions A B and C was soluble after removal of SDS according to method 2 These fractions each of which contained about 2 mg per 100 ml of protein were tested according to the standard neutralization test None of the fractions were found to have any neutralizing capacity The addition of goat anti rabbit H chain serum to the mixtures had no effect on virus survival

The H and L chain fractions The pooled insoluble parts of the A B and C fractions rendered soluble in glycine HCl buffer at pH 3 0 will be referred to as the H chain fraction The H chain and the "L chain fractions and a mixture of these in Tris HCl buffer at pH 3 0 were dialysed over night against 0 01 M Tris buffer at pH 8 0 The L chain fraction and the solution of H chain and L chain fractions were clear after the dialysis and the H chain" fraction was slightly opalescent After the dialysis the preparations were tested for neutral

izing capacity The results are given in Table 2 None of the preparations revealed any neutralizing effect No reduction in virus survival was produced by addition of goat anti rabbit H chain serum to the virus H chain mixture or by addition of anti rabbit L chain serum to the virus L chain mixture However although the reconstituted solution of H chain and L chain fractions by itself had no demonstrable activity at the concentration tested a binding to the virus particles was demonstrated by a decrease of the surviving fraction of virus after addition of the goat anti rabbit H chain serum

TABLE 2

Neutralizing Capacity at pH 8 0 of H Chain Fraction L Chain Fraction and a Solution of H and L Chain Fraction

Original mixture		Fraction of virus escaping inactivation after addition of		
Fractions of IgG mg/100 ml	Adenovirus PFU/ml	buffer	goat anti H	goat anti L
H chains 30	7 3 × 10^5	1 0	1 0	1 0
L chains 33	1 1 × 10	1 0	1 0	1 0
H+L chains 24+22	7 3 × 10	1 0	3 2 × 10	4 3 × 10

H chains were solubilized in glycine HCl buffer at pH 3 0 and subsequently dialysed against 0 01 M Tris buffer at pH 8 0 H chains and L chains were mixed in glycine HCl buffer at pH 3 0 and subsequently dialysed against Tris at pH 8 0 The L chains were dialysed against Tris pH 8 0 All solutions were mixed with virus at pH 8 0

1 0 is recorded for surviving fractions with a PFU count ± 25 per cent of the control

TABLE 3

Neutralizing Capacity at pH 3 0 of H Chain Fraction L Chain Fraction and a Solution of H and L Chain Fractions

Original mixture		Fractions of virus escaping inactivation after addition of
Fractions of IgG mg/100 ml	Adenovirus PFU/ml	buffer
H chains 30	2 6 × 10	2 3 × 10
L chains 33	2 6 × 10^6	1 0
H+L chains 24+22	2 6 × 10	7 0 × 10^{-}

The isolated fractions were dissolved in glycine HCl buffer at pH 3 0 Virus preparations were suspended in Tris HCl at pH 3 0 All mixtures and the virus controls were incubated at pH 3 0 for 1 hour and subsequently at pH 8 0 for another 2 hours before being assayed

Substantial evidence of antibody activity was observed when the H chain fraction the L chain fraction and a mixture of these in glycine HCl at pH 3 0 were tested for neutralizing activity according to the low pH test *i e*, fractions and virus were mixed and incubated at pH 3 0 for 1 hour The results of these experiments are given in Table 3 The H chain fraction had a pronounced effect At the concentration tested the activity of the H chain *fraction was of the same* order of magnitude as that of the native IgG at pH 8 0 The L chain fraction at molar concentration about twice that of the H chain had no effect on virus survival The H + L chains solution produced marked neutralization though weaker than that produced by the H chain fraction alone

DISCUSSION

The results obtained by separation of heavy and light chains from rabbit IgG according to the method of *Karush & Utsumi* (1964) and the sedimentation analysis agreed well with those reported by these authors As the possibility of minor H and L chain contaminations cannot be excluded by the technique used or by any other chain separation method we have prefered to use the terms H chain and L chain fraction respectively

The precipitate obtained on removal of SDS from the H chain fractions was soluble in glycine HCl at pH 3 0 This is in agreement with the finding (*Hansson* 1968) that IgG molecules aggregated by freezing were converted to monomeres in glycine HCl at pH 3 0 In our system the dissolved H chain fraction could be tested at pH 3 0 for *virus neutralizing activity* as *previously described* (*Kjellen* 1966) involving an advantage since the fractions could be assayed not only for binding capacity but also for biological activity

The L chain fraction did not inactivate virus at pH 8 0 nor at pH 3 0 In view of the results given in a previous study (*Kjellén* 1964) which demonstrated that F ab fragments had lost most of the neutralizing capacity inherent in the native IgG this inability of the L *chains was expected*

The capacity of the H chain fraction to neutralize virus at pH 3 0 was of the same order of magnitude as that of untreated IgG at pH 8 0 The results showed that under the present experimental conditions the H chain fraction of the antibodies was responsible for the neutralizing effect

The H chain fraction possessed viral neutralizing power in glycine solution at pH 3 0 It was not active at pH 8 0 This suggests that the conformation of the H chains and/or the virus particles varies with pH The effect may also depend on the presence of glycine

It could be argued that the neutralizing effect achieved by *the* H chain might be caused by a contamination of L chain *However the*

presence of L chain as a contaminant has not been demonstrable by the analysing methods used The possibility that the virus specific neutralizing antibody constitutes a very small per cent of the IgG tested and that these molecules for some reason should exclusively prove resistant to separation seems not plausible

The specificity of the H chain at low pH requires further experiments which are under way

When the H chain and L chain fractions were mixed with virus at pH 3 0 the effect was not so strong as when only H chain fraction and virus were mixed in corresponding amounts The IgG used for fractionation contained a heterologous population of antibodies For example the capsid of the adenovirus particle itself contains several different antigens but whether one or another of these stimulates the specific neutralizing antibodies is still a controversial question (*Wilcox & Ginsberg* 1963) (*Kjellen & Pereira* 1967) In the mixture at pH 3 0 the H chain the L chain and the virus antigens may compete for sites to combine with Viral combining sites of the H chains may not be interfered with by combination with homologous L chains Combinations between H chains and heterologous L chains may decrease the affinity for the virus This is born out by the finding that the mixture of H chain and L chain fractions dialysed against buffer at pH 8 0 before mixing with virus lacked neutralizing power although the combined fractions remained in solution at this pH Unlike the isolated H chains however the solution of the H and L chain fractions after dialysis against pH 8 0 buffer was found to combine with virus This showed that the L chains can preserve the structure of the H chains and their ability to combine with antigenic sites

SUMMARY

Rabbit IgG against adenovirus type 5 was reduced alkylated and separated in H and L chain fractions The H chain fraction rendered soluble in glycine HCl buffer pH 3 0 retained a virus neutralizing capacity of the same order of magnitude as that of untreated IgG at pH 8 0 The L chain fraction lacked capacity to neutralize virus According to the furnished evidence the L chains protected apparently the structure of the H chains under varying conditions

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STUDIES ON ANTIGEN PREPARATIONS FROM *STAPHYLOCOCCUS AUREUS*

4 *Separation and Purification of Protein A and a Related Precipitinogen*

By

ARNE GROV

Received 9 xii 67

Protein A (antigen A) of *Staph aureus* has been characterized serologically by a precipitation line produced in agar gel with all normal human sera (7 8) Purified preparations seem however to contain more than one serologically active component (14 13 10) The protein A preparation described by *Oeding et al* (13) formed two precipitation lines in agar with rabbit antisera against the homologous strain sensitized tanned sheep erythrocytes to agglutination (13) and inhibited agglutination of *Staph aureus* strains (6 5) The preparation is solely of a protein nature No separation of the various activities has been obtained either on column chromatography or electrophoresis Neither was any marked heterogeneity observable on ultracentrifugation (3) On the other hand the results from experiments including absorption chemical modification and enzyme digestions (2) strongly indicate that the different serological activities are due to separate molecules

This paper presents a method of separating the serologically active components of a protein A preparation and a comparison of the molar amino acid composition of the two precipitinogens the preparation contains

MATERIALS AND METHODS

The *Staph aureus* strain Cowan I used throughout these experiments was grown for 18 hrs on nutrient agar

The antigen material containing protein A was prepared according to the procedure described in (4) Since this material exhibits several serological activities it is here referred to as *crude protein A*

Twenty normal human sera were tested for antibody content pooled centrifuged and stored in portions at —28 C until use The rabbit antiserum against strain

The analyses by the automatic amino acid analyzer were carried out at The Department of Clinical Biochemistry Haukeland Sykehus Bergen For this I am much indebted to Mag scient *J Boe*

Cowan I was the same as in previous experiments (4 13) The techniques of ring test agar gel precipitation indirect haemagglutination antibody and antigen absorption and immune electrophoresis also the methods of hydrochloric acid hydrolysis and qualitative paper chromatography were carried out as before (4 13) Bacterial agglutination was performed on slides as described by *Oeding* (12)

Inhibition studies were carried out by making serial twofold dilutions of the antigen in 0 15 ml volumes To each tube was added 0 15 ml of serum at a 16 times lower dilution than its agglutination titre After incubation the mixtures were examined by agglutination on slides against whole Cowan I cells

The equivalence zone of precipitation was determined by adding increasing amounts of crude protein A to a series of tubes with constant volume of the human serum pool diluted 1/2 in saline After incubation and centrifugation the supernatants were tested for free antigen and antibody

Unless otherwise stated all incubations were carried out at 37 C for 2 hrs and at 4 C overnight

Complement fixation tests were carried out using active normal rabbit sera as well as guinea pig sera as complement source Since normal guinea pig sera precipitate protein A (9) these sera had to be neutralized with protein A prior to the complement fixation test The neutralization was performed as described above for the determination of the equivalence zone in normal human serum except that incubation at 37 C was omitted.

Washed sheep erythrocytes were suspended in saline at a concentration of 3 per cent In titration of the complement an excess of haemolysin (rabbit antiserum to sheep erythrocytes) was employed and the haemolysin was then titrated using 2 units (100 per cent) of complement Two units of complement and 2 5 units of haemolysin were employed in the final tests

The immune serum and normal human serum were diluted twofold (from 1/10) in 0 2 ml volumes To each tube 0 2 ml of diluted antigen and 0 2 ml of complement (2 units) were added The mixtures were incubated at 37 C for 30 mins 0 4 ml of a 1 5 per cent suspension of sensitized erythrocytes was added and the mixtures were left for another 30 mins at 37 C The degree of haemolysis was estimated visually

The quantities of amino acids in hydrolysates of purified preparations were determined according to *Moore et al* (11) by an automatic amino acid analyzer (Beckman Spinco Model 120 B)

EXPERIMENTS AND RESULTS

In the complement fixation test crude protein A was found to be anticomplementary at a concentration of 1 mg per ml At lower concentration complete haemolysis indicated that protein A in the presence of rabbit immune serum or normal human serum did not fix complement Identical results were obtained both with rabbit and guinea pig complement

Of the two precipitinogens in crude protein A only one (protein A) forms a precipitate with normal human serum This property can be utilized in separating the two antigens

The equivalence zone of precipitation in normal human serum was obtained at a mixture ratio of about 300 μg crude protein A to one ml serum (½) The protein A antigen was quantitatively precipitated whereas the antigen giving the second line in agar gel with rabbit antiserum remained in the supernatant This latter antigen is according to previous studies (4) also of a protein nature and it is designated *protein B*

The supernatant at the equivalence zone did not sensitize tanned erythrocytes or agglutinate tanned sheep erythrocytes sensitized with

crude protein A Therefore the sensitizing substance of crude protein A as well as its corresponding antibody was apparently precipitated

In order to dissociate the components of the precipitate it was suspended in saline heated for 5 mins in a boiling water bath and centrifuged for 30 mins at 27 000 × g Both protein A and the sensitizing substance were demonstrated in the resulting supernatant When this experiment was carried out with rabbit antiserum the same co precipitation occurred

A *Isolation of Protein A*

To separate the sensitizing substance from protein A and protein B a saline solution of crude protein A was absorbed with tanned sheep erythrocytes until the sensitizing substance was completely removed (13) The absorbed solution (Table 1 supernat 1) was then mixed with normal human serum in an amount corresponding to a slight excess of antibody (0 5 mg antigen material per ml of undiluted serum) After incubation the mixture was centrifuged for 30 mins at 27 000 × g

TABLE 1

Scheme for the Separation Procedure

Saline of crude prot. A

Abs. w tanned sheep erythr
Centrifug

Supernat 1 — Sediment (sensit. tanned sheep erythr)

Supernat 1: Incub w human serum
Centrifug

Supernat 2 — Precipitate

Supernat 2: Incub w rabbit antiserum
Centrifug

Precipitate: Washing resusp saline
Heating 5 mins 100 C
Centrifug

Supernat 4 — Precipitate — Supernat 3 — Sediment

Supernat 3: *Protein A*

Precipitate: Washing resusp saline
Heating 5 mins 100 C
Centrifug

Supernat 5 — Sediment

Supernat 5: *Protein B*

The supernatant (Table 1 supernat 2) was tested for antibody and antigen By ring test and agar precipitation a small amount of protein

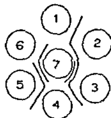


Fig 1

Reaction in agar gel diffusion of crude protein A (wells 3 and 5) protein A (well 2) and protein B (well 6) against Cowan I antiserum (well 7) and normal human serum (wells 1 and 4)

A antibody in addition to protein B was demonstrated The super natant was unable to sensitize tanned sheep erythrocytes but agglutinated tanned erythrocytes sensitized with crude protein A

The precipitate (protein A—anti protein A) was washed 3 times in cold saline resuspended in saline and heated in a boiling water bath for 5 mins The undissolved material was removed by centrifugation at 55 000 × g for 1 hr in a preparative ultracentrifuge When tested in agar the supernatant (supernat 3) showed the protein A line with human serum as well as with rabbit antiserum However a trace of protein B was still likely to be present and protein A of supernat 3 was therefore reprecipitated in normal human serum and the whole procedure repeated

After this complete fusion of the lines which supernat 3 produced against normal human serum and antiserum was obtained (Fig 1) No sensitizing ability was demonstrated in the isolated protein A This is in accordance with the test showing complete absorption of sensitizing substance from the solution of crude protein A (Table 2) Purified protein A did not inhibit agglutination of Cowan I bacteria in amounts up to 1 mg whereas 40 µg of the crude protein A preparation completely inhibited this reaction in Cowan I antiserum No complement fixation was observed

B *Isolation of Protein B*

The second supernatant (Table 1 supernat 2) was mixed with Cowan I antiserum (1 ml serum per mg of crude protein A) and incubated The precipitate formed was isolated and further treated as above The *antigen fraction* was *reprecipitated with* normal human serum and then with Cowan I antiserum The final supernatant (supernat 5) showed the protein B line only on agar gel diffusion (Fig 1) No line could be observed against normal human serum and no sensitization of tanned sheep erythrocytes was obtained (Table 2) Inhibition experiments with the isolated protein B showed no effect on the bacterial agglutination The complement fixation test was also negative

TABLE 2

Results of Serological Tests. Crude and Purified Preparations Tested with Normal Human Serum and Rabbit Antiserum

	Agar precipitation Lines with		Indirect haemaggl.	
	Human serum	Cowan I antiserum	Human serum	Cowan I antiserum
Crude protein A	protein A	protein A protein B	+	+
Crude protein A abs. w. TSF	protein A	protein A protein B	—	—
Protein A (Supernat. 3)	protein A	protein A	—	—
Protein B (Supernat. 5)	—	protein B	—	—

TSE = tanned sheep erythrocytes

C. *Characterization*

On immunoelectrophoresis no difference in the electrophoretic mobility of the two isolated antigens could be observed. Both moved identically with crude protein A (4).

TABLE 3

Molar Ratios of Amino Acids in Hydrolysates of Purified Precipitinogens Using Lysine as Unity

Hydrolysates of	Lys	Asp A	Ser	Glu A	Gly	Thr	Ala	Pro	Val	Leu
Protein A	1.0	1.8	2.3	2.4	1.7	0.5	1.5	0.6	0.7	1.5
Protein B	1.0	1.8	9.2	3.4	4.8	1.0	3.6	0.6	0.8	2.6

Portions of protein A and protein B were hydrolysed in 3 N HCl at 100° C for 3 hrs and in 6 N HCl at 105° C for 18 hrs. The hydrolysates were examined by paper chromatography and by an automatic amino acid analyzer. Both antigens contained the same amino acids as the crude preparation viz. lysine, aspartic acid, serine, glutamic acid, glycine, threonine, alanine, proline, valine and leucine. No other substances could be observed in the chromatograms. The quantitative analyses however demonstrated as shown in Table 3 a marked difference between the two antigens with regard to the molar ratios of the neutral amino acids. The molar ratios of acidic to basic amino acids were the same in both antigens.

DISCUSSION

Absorption of a solution of crude protein A with tanned sheep erythrocytes removed the sensitizing antigen whereas the two precipitinogens protein A and protein B remained in solution As only protein A forms a precipitate with normal human sera protein B remained in the supernatant and was precipitated from this by addition of rabbit antiserum Owing to the relatively high thermo resistance of both precipitinogens (4) the precipitates could be exposed to a temperature which not only broke the linkage between antigens and antibodies but also made the latter insoluble Chromatographic examination of hydrolysates of the isolated precipitinogens showed the same amino acids as previously demonstrated in crude protein A (4) and none of the other amino acids found in whole proteins such as γ globulins This indicates that the denatured antibodies were quantitatively sedimented by the high speed centrifugation employed

The only difference observed between protein A and protein B was the molar ratios of their amino acids Since both antigens apparently possess the same molar ratios between positively and negatively charged amino acids lysine aspartic acid glutamic acid the net charges of the molecules are likely to be equal and explain the identical electrophoretic mobility As the two antigens also have very similar molecular weights (3) it is understandable that we have not succeeded in separating the antigens by other methods In our experience protein B has the same distribution among staphylococci as protein A

The results of the serological tests indicate complete separation of the various serological activities of crude protein A In both isolated protein A and protein B only the precipitating ability was observed each preparation producing a single line in agar with rabbit antiserum

The assumption that protein A is both a precipitinogen and an agglutinogen (8 14 6 10) has been based on experiments in which protein A preparations inhibit bacterial agglutination when added to antisera Contrary to crude protein A the separated protein A and protein B did not inhibit bacterial agglutination in the present experiments This indicates that neither protein A nor protein B is responsible for the ability of crude protein A to inhibit agglutination of staphylococci A further examination of this question will be reported in a subsequent paper

The precipitation line produced by protein A and normal human serum in agar is always diffuse fusing with in angle instead of an arc with the corresponding line against rabbit antiserum The latter line is sharp and well defined two such lines forming the characteristic arc on fusion Recently a report by *Forsgren & Sjoquist* (1) indicates that the binding of protein A to the γG fraction of normal human sera is due to a pseudo immune reaction

SUMMARY

A method for purifying protein A and protein B of *Staph aureus* is described The sensitizing antigen of the crude preparation was removed by absorption with tanned sheep erythrocytes Protein A was precipitated quantitatively with normal human serum and protein B from the resulting supernatant by addition of rabbit antiserum Chemically no qualitative difference between the isolated precipitinogens or between these and the crude preparation was observed The molar ratios of most neutral amino acids were found to be different in the two precipitinogens but the net charge was equal Serologically protein A as well as protein B showed precipitating ability only

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STUDIES ON ANTIGEN PREPARATIONS FROM *STAPHYLOCOCCUS AUREUS*

5 *Inhibition of Bacterial Agglutination by Protein A Preparations*

By

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Received 9 xii 67

The protein A precipitinogen has been demonstrated in *Staph aureus* cell wall preparations (11 6 5) and thus shown to be a surface component *Jensen* (7) suggested that precipitation of protein A as well as agglutination of staphylococci by normal human sera was due to the same antibody This question has also been discussed by others (1 11 8 6) and the general opinion seems to be that protein A is both a precipitinogen and an agglutinogen This assumption has been based on the observation that protein A preparations inhibit bacterial agglutination when added to rabbit antisera and normal human sera and also that they induce production of bacterial agglutinins in rabbits

Since the protein A preparation described in (4) as well as that of *Lofkvist* (8) is antigenically heterogeneous the present study was undertaken to re-examine the validity of the above mentioned assumption

MATERIALS AND METHODS

Strains Seventeen *Staph aureus* three *Staph epidermidis* and two micrococcal strains were employed All strains were grown for 18 hrs on nutrient agar at 37 C

Antigen preparations Crude protein A purified protein A and purified protein B were prepared from *Staph aureus* strain Cowan I as described in (3)

Sera Rabbit antisera against the *Staph aureus* strain Cowan I the *Staph epidermidis* strains and the micrococcal strains were produced according to the procedure described by *Oeding* (9)

Crude protein A and tanned sheep erythrocytes (TSF) sensitized with crude protein A were also injected intravenously The immunization was performed in series each consisting of one injection on three consecutive days with an interval of f days between each series The rabbits were bled one week after the last injection Crude protein A was given in increasing amounts 0 5 ml of 1 mg per ml saline in the first series 1 ml of 2 mg per ml in the second and a further two series of 1 ml containing 4 mg antigen per ml The antiserum obtained is referred to as *serum crude protein A* Sheep erythrocytes were tanned and sensitized with crude protein A as previously described (10) and then injected in 3 series During the first and second series 6 × 1 ml of a 10 per cent saline suspension of erythrocytes was injected and in the last series 3 × 1 ml of a ?5 per cent suspension The antiserum obtained is referred to as *serum TSF—crude protein A* Before use it was absorbed with TSF

Immunization with purified protein A in complete *Freund*s adjuvant was performed according to the procedure described by *Löfkvist* (8) Three doses of 2 0 µg

protein A were injected subcutaneously into the ne k The serum obtained is referred to as *serum protein A*

In addition to the pool of 20 normal human sera used earlier (3) some individual normal human sera were employed

Mercaptoethanol treatment of sera was performed as before (2) *Bacterial agglutination* was carried out on slid s (9) and the performance of *ring test precipitation agar precipitation* and *indirect haemagglutination* was as earlier (4 10)

Experiments in *inhibition of bacterial agglutination* were carried out as follows Serial twofold dilutions of the antigen were made with saline in 0 15 ml volumes and 0 15 ml of a serum dilution 16 times lower than its agglutination titre was added to each tube The mixtures were incubated at 37 C for 2 hrs and at 4 C overnight After centrifugation the clear supernatants were titrated for bacterial agglutinins

RESULTS

Agar precipitation bacterial agglutination and indirect haemagglutination titres of the sera examined so far are summarized in Table 1 In the pre immune sera only low titres of bacterial agglutinins (titres < 1/10) were demonstrated Precipitating or haemagglutinating anti bodies were *not found* *Immunization* with crude protein A induced production of precipitins against protein A and protein B bacterial agglutinins and agglutinins to tanned sheep erythrocytes sensitized with crude protein A Only the latter type of antibodies was observed in serum TSE crude protein A Protein A and protein B precipitins and bacterial agglutinins were not produced Serum protein A revealed production of protein A precipitins only No production of agglutinins was observed

TABLE 1

Titres of Antisera and Normal Human Sera in Different Serological Tests

	Antisera to Crude protein A	TSE crude protein A	Protein A	Strain Cowan I	Pooled normal human sera
Agar precipitation w protein A (0 5 mg/ml)	20	<1	30	40	40
Agglutination w Cowan I bacteria	2560	<10	<10	5120	160
Indirect haemaggl w TSE treated w crude protein A	640	160	<10	20480	10240

Reciprocals of highest serum dilution which gave positive reaction
TSE *Tanned sheep erythrocytes*

In addition to strain Cowan I serum crude protein A agglutinated the 16 other *Staph aureus* type strains examined at dilutions ranging from 1/40 to 1/1280 After absorption with one of these strains (3647 which was agglutinated at a 1/40 dilution of the serum) only strain Cowan I was agglutinated The titre was 1/400 The remaining agglutinins could not be affected by further absorption with strain 3647 or with

TABLE 2

Titres of Antiserum to Crude Protein A Unabsorbed and Absorbed with Various Antigen Preparations

	Tests and test materials		
	Agar precipitation Crude protein A (0 5 mg/ml)	*Agglutination* Cowan I bacteria	*Indirect ha maggl* TSF treated w crude protein A
Unabsorbed	20	2560	640
Absorbed with			
1 Crude protein A	–	–	–
2 TSF sens w crude protein A	20	2560	–
3 Crude protein A from which sens subst has been removed		–	640
4 Purified protein A	(Protein B line)	2560	640
5 Purified protein B	(Protein A line)	2560	640

Reciprocals of highest serum dilution which gave positive reaction
TSE Tanned sheep erythrocytes

any of the other 15 strains but absorption with Cowan 1 removed all agglutinins

Absorption with either crude protein A or crude protein A from which sensitizing substance had been removed exhausted Cowan I antiserum serum crude protein A and normal human serum for agglutinins against Cowan I bacteria Purified protein A protein B or TSE sensitized with crude protein A did not affect the bacterial agglutination (Table 2)

Forty μg crude protein A completely inhibited agglutination of Cowan I bacteria in Cowan I antiserum as well as in serum crude protein A In a corresponding test with normal human serum 10 μg crude protein A gave complete inhibition Purified protein A and protein B did not inhibit agglutination

None of the three *Staph epidermidis* strains or the two micrococcal strains produces the protein A line in agar In inhibition experiments with these strains and their homologous antisera crude protein A had no effect on their agglutination titre

To study the different activities of serum crude protein A as a function of antigen material added and also to estimate the agglutination titre at the equivalence zone of precipitation crude protein A was added to a constant volume of the serum (0 5 ml 1/10) in amounts varying from 10 μg to 800 μg After incubation and centrifugation all the supernatants were titrated for bacterial agglutinins against strain Cowan I and tested for free precipitinogens by ring test and agar gel diffusion The results are illustrated in Fig 1 No redu n of the bacterial agglutination titre could be observed at the equi ale one of precipi

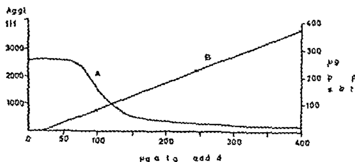


Fig 1

Activities of supernatants after addition of increasing amounts of crude protein A to 0.5 ml (1/10) of its antiserum *Curve A* Agglutination titre with Cowan I bacteria (reciprocals of highest dilution which gave agglutination) *Curve B* Precipitating substance as determined by ring test

tation Significant reduction was not demonstrated until about 100 µg antigen material was added corresponding to about 75 µg antigen material in excess of the equivalence of precipitation

As the agar precipitation test is less sensitive than the ring test the first tube showing excess precipitinogen on agar diffusion was that to which 100 µg antigen had been added *viz* the same supernatant that showed the first significant reduction of the agglutination titre

To prepare a serum containing bacterial agglutinins only crude protein A was added to its antiserum in an amount corresponding to the equivalence zone of precipitation followed by incubation and centrifugation Judging from the precipitation tests the resulting supernatant did not contain or precipitate protein A but agglutinated Cowan I bacteria at a total dilution of 1/2560 Purified protein A added to this supernatant gave the same precipitation titre with normal human serum as an equal concentration of purified protein A in saline

No correlation between precipitation and bacterial agglutination titres could be observed with the individual normal human sera tested There was a relatively small variation in the precipitation titres (1/40–1/80) while the bacterial agglutination titres varied from 1/10 to 1/5120 Some sera showing the highest precipitation titre showed the lowest agglutination titre and others vice versa

Treatment of serum crude protein A with mercaptoethanol did not reduce either precipitation or bacterial agglutination titres indicating that both types of antibodies were 7S γ globulins

DISCUSSION

The crude protein A preparation inhibited agglutination of *Staph aureus* strains in rabbit antisera and normal human sera and induced production of agglutinins when injected into rabbits Since crude protein A did not affect the agglutination of the three *Staph epidermidis*

strains or the two micrococcal strains tested the presence of an unspecific inhibitor is not likely

The antiserum to crude protein A agglutinated 17 *Staph aureus* strains but only Cowan I bacteria were agglutinated after absorption with one of the other strains. This shows that the serum contains agglutinins common to *Staph aureus* and in addition agglutinins specific to strain Cowan I. Consequently crude protein A contains at least two different agglutinogens.

Immunization with tanned sheep erythrocytes sensitized with crude protein A induced antibody response to the sensitizing substance but neither precipitins nor agglutinins to *Staph aureus* strains were produced. The results of experiments with serum TSE crude protein A in addition to those obtained by absorption of serum crude protein A with sensitized tanned erythrocytes (Table 2 absorption 2) should exclude the possibility that the sensitizing and agglutinating antigens are identical.

Contrary to the findings of *Löfkvist* (8) no reduction of the bacterial agglutination titre was observed in the equivalence zone of precipitation. About 80 times the amount of antigen material used to remove the precipitins from serum crude protein A was needed to obtain complete inhibition of agglutination. The bacterial agglutination in serum crude protein A was not affected by any of the two isolated precipitinogens protein A and protein B. This accorded with the immunization experiments with purified protein A which showed no production of agglutinins (serum protein A). Furthermore bacterial agglutinins did not seem to inhibit precipitation of protein A or protein B.

All these findings point to the conclusion that neither protein A nor protein B exhibits the properties of an agglutinogen and that the ability of crude protein A preparations to inhibit bacterial agglutination is due to the presence of other antigens. These antigens seem to be minor parts of the crude protein A preparations and may thus like the sensitizing substance be regarded as contaminants of the precipitating material.

Accordingly such contaminants can be expected to vary in amounts from one preparation to another leading to discrepancies in test results.

SUMMARY

The question of whether or not the protein A precipitinogen is also an agglutinogen has been studied. Crude protein A preparations known to contain two precipitinogens and an antigen which sensitizes tanned sheep erythrocytes are effective inhibitors of bacterial agglutination in *Staph aureus* antisera and normal human sera.

Antiserum to crude protein A to tanned sheep erythrocytes sensitized with crude protein A and to isolated protein A precipitinogen were produced. The content of antibodies in these sera and the results of

absorption and inhibition experiments demonstrated the existence of specific agglutinogens in crude protein A. The agglutinogens are distinct from the other antigens in the preparation (protein A, protein B and sensitizing substance), are present in relatively small amounts and were removed by separation and purification of the precipitinogens.

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ANTIGENIC PROPERTIES OF VARIOUS PREPARATIONS OF *NEISSERIA GONORRHOEAE* ENDOTOXIN

By

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Compared to the extensive investigations on endotoxic lipopolysaccharides from Gram negative bacilli very little is known of *Neisseria gonorrhoeae* endotoxin In 1944 *Boor & Miller* (1) prepared a glycolipid from gonococci by extraction with trichloroacetic acid The glycolipid formed an opalescent colloidal suspension in water was toxic for mice and antigenic in rabbits *Tauber & Garson* (17) in 1957 extracted endotoxin from gonococci by treatment of dried bacterial cells with alkali The preparation had a low toxicity for mice These authors later prepared a highly toxic endotoxin by extraction with phenol water (18) On the basis of the chemical composition they called the endotoxin a lipopolysaccharide phosphoric acid ester The serological properties were not studied

Alkali extracts from gonococci have been studied by *Thomas & Mennie* (20) *Chanarin* (2) and recently by the author (12 13) Erythrocytes sensitized with this extract became agglutinable with antisera to gonococci Extraction of Gram negative bacilli with alkali is known to release the somatic O antigen (endotoxin) Erythrocytes sensitized with this antigen agglutinate in the presence of homologous antisera (4 16)

The present work was started with the intention of seeing whether or not the erythrocyte sensitizing antigen of the alkali extract was identical to or part of the endotoxin of gonococci A number of extracts have been prepared by methods frequently employed for the preparation of endotoxins The serological cross reactivity and some physical and biological properties of the extracts are described

MATERIALS AND METHODS

N gonorrhoeae strain Strain 8551/64 was employed Cultivation and harvesting were performed as described previously (12) The bact[illegible] were washed three times with 10 volumes of saline before extraction

Antisera Rabbit antisera to whole gonococci (an[illegible] were prepared as described earlier (12) The antiserum against sensitized [illegible]mal sheep [illegible] erythrocytes (anti SNSE) was that previously employed (13) This a[illegible]um [illegible] prepared by

immunization of rabbits with sheep erythrocytes sensitized with the alkali extracted antigen preparation and was absorbed with sheep erythrocytes before use

Extraction methods The bacteria were extracted with alkali as described previously (12) The extract is referred to here as *alkali endotoxin*

Extraction with 0 25 M trichloroacetic acid (TCA) was performed at 4 C for 4 hrs Four ml TCA was employed for each g of bacteria (wet weight) Before centrifugation the suspension was neutralized with NaOH

Another batch of gonococci was extracted by heating The bacteria were suspended in 4 ml of saline per g of wet bacteria and the mixture was kept in a boiling water bath for 1 hr

Gonococci extracted either with TCA or by heating were centrifuged at 4 000 × g for 30 mins and the supernatants were further handled as described below for aqueous ether endotoxin The preparations obtained are designated *TCA endotoxin* and *heat endotoxin*

Aqueous ether endotoxin was prepared according to the procedure of *Ribi et al* (15) *with some modifications Gonococci* were suspended in 4 ml of saline and 8 ml of diethylether per g bacteria in stoppered flasks The suspensions were kept at 4 C for 48 hrs and were occasionally shaken After centrifugation at 4 000 × g for 30 mins the water layer was withdrawn and 2 volumes of cold acetone were added The precipitate was collected by centrifugation dissolved in saline and centrifuged at 4 000 × g for 30 mins Any sediment was discarded The dialyzed supernatant constituted the crude aqueous ether endotoxin Part of it was withdrawn for dry weight estimation the rest was adjusted to the desired concentration and kept at 4 C

Phenol water endotoxin was prepared essentially as described by *Tauber et al* (18 19) Gonococci were extracted with 4 ml of water and 4 ml of 90 per cent phenol per g wet weight at 37 C for 20 mins After centrifugation the water face was withdrawn and dialyzed and 2 volumes of cold acetone were added The precipitate obtained after centrifugation was taken up in water and the lipopolysaccharide was separated from other constituents by ultracentrifugation at 105 000 × g for 1 hr in a Spinco Model L ultracentrifuge The sediment was re dissolved in water and the ultracentrifugation was repeated once Finally the sediment was taken up in water to yield an opalescent solution The volume was adjusted to 1 mg of phenol water endotoxin per ml and the preparation was kept at −20 C

Serological methods The indirect haemagglutination test the indirect haemolysis test and *the ring precipitation test* were performed as described earlier (12 13) *Sheep erythrocytes were sensitized either with 100 µg of alkali endotoxin or with* 10 µg of alkali treated phenol water endotoxin per ml of 1 per cent erythrocytes The phenol water endotoxin had been treated with 0 02 N NaOH at 37 C for 18 hrs (see below)

Tests for inhibition of haemagglutination and haemolysis were performed as follows Two fold dilutions of the preparations were prepared in phosphate buffered saline pH 7 2 in 0 2 ml volumes To each tube was added 0 2 ml of antiserum containing 8 agglutinating units After incubation for 1 hr at 37 C 0 2 ml of 0 5 per cent suspension of sensitized erythrocytes was added The results were recorded the next day Minimal inhibiting dose (MID) of the preparation was defined as the least amount (in µg) which completely inhibited the agglutination In some experiments the erythrocytes were centrifuged after the agglutination had been read washed once and resuspended in saline Addition of complement incubation and reading followed the procedure of the indirect haemolysis test (12) Appropriate controls were included

Absorption of the antiserum was performed by incubation with samples of the endotoxin preparations at 4 C *for 4 hrs followed by centrifugation*

Bioassays for endotoxic activity Endotoxin to be employed for bioassays was diluted in commercial pyrogen free saline Adult rabbits were prepared for the local Shwartzman reaction by intradermal injection into the skin of the abdomen of 100 µg of endotoxin in 0 2 ml volumes followed 24 hrs later by intravenous *injection of 100 µg of the preparation*

The epinephrine skin test was performed by intravenous injection of 100 µg of endotoxin into rabbits followed 30 mins later by intradermal injection into the shaved abdomen of 100 µg 1 epinephrine bitartrate (Calbiochem) The animals were inspected for dermal lesions 24 hrs after the last injection

Other methods Oxidation with periodate was performed by mixing equal volumes

of a 0.2 per cent solution of aqueous ether endotoxin and 0.04 M sodium metaperiodate. The mixture was incubated in the dark at 20° C overnight, dialyzed against buffered saline and tested for serological activity.

Enzyme digestions of aqueous ether endotoxin were performed with crystalline trypsin (Trypure Novo), papain (Sigma Chemical Co.) and pronase B Grade (Calbiochem.). Digestion with trypsin was performed in 0.1 M tris buffer pH 7.8. The following ratios of enzyme to substrate were employed: 1:1, 1:10 and 1:50 (W/W). Treatment with papain was carried out in 0.1 M phosphate buffer pH 7.4 with the addition of 0.01 M cysteine and 0.002 M EDTA, the enzyme to substrate ratio being 1:10. Digestion with pronase was performed with an enzyme to substrate ratio of 1:10 in phosphate buffered saline pH 7.4. All digestions were performed at 37° C for 18 hrs followed by inactivation of the enzymes at 100° C for 5 mins. Untreated endotoxin was included as a control and the enzyme activity was investigated by digestion of coagulated ovalbumin.

Dialysis was performed in the cold in cellophane tubes against running tap water for 2 days and finally against distilled water for 1 day.

Optical densities were measured with a Unicam sp 500 Spectrophotometer with 1 cm cells.

EXPERIMENTS AND RESULTS

Physical Properties of the Preparations

The preparation of aqueous ether and TCA endotoxins deserve special comments. After centrifugation of aqueous ether extracted bacteria 4 layers were recognized. Reckoned from the top they were the ether layer, solids at the ether saline interphase, the saline layer which contained the endotoxin, and solids at the bottom of the tube. The presence of endotoxin was determined by the haemagglutination inhibition test as described below.

Centrifugation of the suspension of bacteria in TCA after the extraction period was finished gave a limpid supernatant. After neutralization and dialysis the supernatant did not react serologically. On the other hand, the endotoxin appeared in the supernatant when the acid was neutralized before centrifugation. It thus seemed that TCA had precipitated the extracted endotoxin.

The yield per gram wet weight of bacteria with the various extraction methods varied between 2 and 6 mg and was as a rule highest with the ether extraction method.

The endotoxin preparations formed relatively stable but opalescent solutions. The alkali extract was, however, less opalescent than the other preparations. All preparations were rapidly clarified when alkali was added. After neutralization the opalescence re-appeared but to a lesser degree than before.

The endotoxin of the various preparations was precipitated with 2 volumes of acetone and could also be precipitated with 4 volumes of ethanol. The aqueous ether endotoxin was also precipitated when hydrochloric acid was added until the pH of the extract was 4.8.

The preparations were partially clarified by centrifugation at 10 000 × g for one hr and a sediment was obtained. The endotoxin was found both in the supernatants and in the sediments. Ultracentrifugation at 60 000 × g for one hr gave a clear supernatant, all the endotoxin

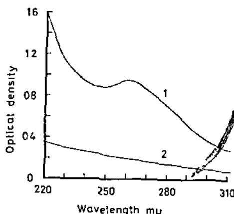


Fig 1

Ultraviolet absorption curve of 0 1 mg per ml in water of crude aqueous ether (1) and phenol water endotoxin (2)

having been sedimented In all serologically active preparations the antigenic determinants described below appeared together

Fig 1 shows the UV absorption curve of the aqueous ether extracted preparation and of phenol water endotoxin The aqueous ether extract contained considerable quantities of UV absorbing materials The peak at 260 mμ indicates the presence of contaminating nucleic acids The TCA *heat and alkali extracts gave essentially similar absorption* curves The absorption curve of phenol water endotoxin which had been purified by ultracentrifugation and washing suggests that this preparation was free of nucleic acids

Lyophilization of the endotoxin preparations resulted in a light grey fluffy material which was strongly hydrophobic It was however readily soluble in 0 05 N NaOH If the solution was neutralized im *mediately the antigen formed aggregates which sedimented spontane* ously Prolonged exposure to alkali for at least one hr resulted in an opalescent solution in which spontaneous sedimentation did not occur

Biological Tests for Endotoxic Activity

Intravenous injection into rabbits of aqueous ether TCA phenol water and heat endotoxin followed by intradermal injection of epin ephrine resulted in a haemorrhagic and necrotic area around the local injection site No lesions appeared in rabbits injected with the alkali extracted preparation Rabbits which were prepared for the local Shwartzman reaction with aqueous ether endotoxin *developed an* haemorrhagic infiltration at the site of injection Thus all the prepara tions except the alkali extract provoked lesions characteristic of endo toxins

Serological Specificities of the Endotoxin Preparations

Sheep erythrocytes were sensitized with alkali endotoxin or with phenol water endotoxin The alkali extract sensitized erythrocytes directly whereas phenol water endotoxin did not After treatment of phenol water endotoxin with alkali less than 5 μg of the preparation sensitized one ml of one per cent erythrocytes to maximal agglutination titres with antisera

Anti Gc and anti SNSE reacted with erythrocytes sensitized with the alkali endotoxin or with alkali treated phenol water endotoxin (Table 1) The titres in the haemolysis test exceeded the haemagglutination titres

TABLE 1
Titres of Anti Gc and Anti SNSE in Indirect Haemagglutination and Haemolysis Test

Erythrocytes sensitized with	Indirect haemagglutination		Indirect haemolysis	
	Anti Gc	Anti SNSE	Anti Gc	Anti SNSE
Phenol water endotoxin	512	64	8192	512
Alkali endotoxin	1024	64	16384	1024

Anti Gc Antiserum to whole gonococci
Anti SNSE Antiserum to erythrocytes sensitized with the alkali extracted preparation

The ability of phenol water endotoxin not treated with alkali and of the alkali endotoxin to neutralize antibodies against sensitized erythrocytes was investigated by inhibition of haemagglutination and haemolysis and by absorption Phenol water endotoxin strongly inhibited the agglutination of correspondingly sensitized erythrocytes (Table 2) The agglutination was also inhibited by the alkali endotoxin but the activity of this preparation was much weaker The agglutination of erythrocytes sensitized with alkali endotoxin was inhibited by alkali endotoxin only not by phenol water endotoxin The results of the test for inhibition of haemolysis agreed with those of the haemagglutination inhibition test

TABLE 2
Minimal Inhibiting Dose (in μg) of Phenol Water Endotoxin and of Alkali Endotoxin Measured by Inhibition of Haemagglutination

Erythrocytes sensitized with	Phenol water endotoxin	Alkali endotoxin
Phenol water endotoxin	0 39	5
Alkali endotoxin	>200	6 2

Anti Gc was absorbed with alkali and phenol water endotoxin respectively The activity against sensitized erythrocytes of the absorbed antiserum is seen from Table 3 For the absorption of 0 1 ml of undiluted antiserum either 500 μg of the alkali endotoxin or quantities ranging from 20 to 200 μg of phenol water endotoxin were employed A 1 16 dilution of anti Gc absorbed with phenol water endotoxin no longer reacted with erythrocytes sensitized with the same preparation The activity of the absorbed antiserum against erythrocytes sensitized with the alkali endotoxin was unaffected The results were similar when anti Gc was absorbed with varying amounts of the endotoxin preparation Anti Gc absorbed with alkali endotoxin no longer agglutinated erythrocytes sensitized with the alkali extract The titre with erythrocytes sensitized with phenol water endotoxin decreased from 1 512 to 1 64 Essentially similar results were obtained when the experiments were performed with anti SNSE

TABLE 3

Haemagglutination Titre of Anti Gc after Absorption with Phenol Water Endotoxin and Alkali Endotoxin Respectively

Erythrocytes sensitized with	Titre after absorption with Phenol water endotoxin	Alkali endotoxin	Unabs control
Phenol water endotoxin	<16	64	512
Alkali endotoxin	1024	<16	1024

TABLE 4

Minimal Inhibiting Dose (in μg) of Various Endotoxin Preparations Measured by Inhibition of Haemagglutination

Preparations tested	System A	System B
Aqueous ether endotoxin	12 5	6 2
TCA endotoxin	12 5	12 5
Heat endotoxin	6	3 1
Alkali endotoxin	25	6 2
Phenol water endotoxin	0 39	>200

System A and B See text

The results indicated that phenol water endotoxin and alkali endotoxin have at least one antigenic determinant in common (determinant *a*) In addition erythrocytes sensitized with alkali endotoxin acquired a second serological specificity (determinant *b*) whereas phenol water endotoxin was devoid of this specificity The serological activity of determinants *a* and *b* can be measured by inhibition of haemagglutination as follows System A Anti Gc and erythrocytes sensitized with phenol water endotoxin Inhition of agglutination indi

cates the presence of determinant *a* System B Anti Gc absorbed with phenol water endotoxin and erythrocytes sensitized with alkali endo toxin A preparation which inhibits agglutination probably contains determinant *b*

The serological activity of aqueous ether TCA and heat extracted endotoxins is seen from Table 4 The preparations inhibited agglu tination of sensitized erythrocytes both in system A and B thus showing the presence of determinants *a* and *b* The minimal inhibiting dose (MID) of the preparations varied between 3 1 and 25 μg and was essentially similar in both systems Preparations extracted from differ ent batches of bacteria varied to some extent in their antibody neutral izing capacity The ability to inhibit the agglutination both in system A and B was however a constant finding with different endotoxin preparations It is important to note that treatment of the endotoxins with 0 05 N NaOH for one hr at room temperature left the antibody neutralizing capacity of determinants *a* and *b* unchanged

In the ring precipitation test a solution containing one mg per ml of each of the three preparations formed a precipitate up to a dilution of 1 16 with undiluted anti Gc

Serological Activity of Determinants a and b after Oxidation with Periodate and Enzyme Digestion

Aqueous ether endotoxin was subjected to oxidation with periodate and digestion with trypsin papain and pronase and was thereafter tested for inhibition of haemagglutination Oxidation with periodate abolished the reactivity of determinant *a* while the reactivity of deter minant *b* was unaffected Digestion with papain and pronase destroyed the activity of determinant *b* The reactivity of *a* was not changed Digestion with trypsin did not affect the serological activity of deter minants *a* or *b* The serological activity of determinant *a* was thus affected by periodate only and that of determinant *b* by the proteolytic enzymes with the exception of trypsin

DISCUSSION

The physical properties of the endotoxin preparations from gonococci resemble those of similar preparations from other Gram negative bac teria These properties include opalescence the hydrophobic character of lyophilized preparations thermostability sedimentation by high speed centrifugation and clarification by the addition of alkali Endo toxins are considered to form heterodisperse suspensions rather than true solutions in water The endotoxin molecules aggregate to form particles with molecular weights ranging from 1 to 20 million (for review and references see 9) The ease with which the gonococcal endo toxin was sedimented by centrifugation indicates such an aggregation Endotoxin which had been partly purified by repeated washings and

ultracentrifugations was difficult to bring back into homogenous suspension unless treated with alkali It is thus possible that purification in itself increases the tendency to aggregation This property renders further studies of the preparations difficult

When the epinephrine skin test was used as a parameter for *in vivo* activity all the preparations except the alkali extract were found to be biologically active In general alkali-extracted endotoxin are either biologically inactive or have a low toxicity (9) The aqueous ether endotoxin also provoked the local Shwartzman reaction in rabbits The various preparations thus contained the endotoxin of *N gonorrhoeae* but the antigen of the alkali extract had probably been detoxified by the alkali

Alkali extracted endotoxins are known to sensitize erythrocytes directly (4 16) This was also the case with the alkali endotoxin from gonococci Phenol water endotoxin did not adsorb to erythrocytes unless the endotoxin had been pretreated with alkali This finding agrees with observations made by many investigators working with lipopolysaccharides from other Gram negative bacteria including meningococci and Veillonella (10 11 14)

Phenol water endotoxin failed to inhibit the agglutination of alkali endotoxin sensitized erythrocytes Accordingly the acquired antigenic specificity of erythrocytes sensitized with alkali endotoxin is different from that of phenol water endotoxin Alkali extracted endotoxin inhibited the agglutination both of correspondingly sensitized erythrocytes and of erythrocytes sensitized with phenol water endotoxin These findings indicate two different antigenic determinants here designated *a* and *b* Phenol water endotoxin contained determinant *a* and the alkali extracted preparation determinants *a* and *b* Also the aqueous ether TCA and heat extract contained determinants *a* and *b* The serological activity of these extracts was however considerably less than that of phenol water endotoxin In addition to the lipopolysaccharide also other antigens have been demonstrated in endotoxin preparations from Gram negative bacilli (7 21) The different O factors of *Salmonella* are however carried by one and the same molecule (3 8)

Anti SNSF which was obtained by immunization of rabbits with erythrocytes sensitized with the alkali extract contained antibodies to both *a* and *b* The sensitized erythrocytes employed for immunization had thus adsorbed both determinants The agglutination of erythrocytes sensitized with a preparation containing both *a* and *b* with antiserum to each determinant will be investigated

The destruction of determinant *a* by oxidation with periodate and its resistance to the action of proteolytic enzymes are indicative of polysaccharide nature of this determinant Determinant *b* is probably a protein since it was resistant to the action of periodate and was destroyed by digestion with papain and pronase The lack of determinant *b* in the phenol water endotoxin is in accordance with the findings

of *Tauber & Garson* (18) to the effect that this endotoxin is composed of lipid and polysaccharide and is essentially protein free Thus *a* must belong to the polysaccharide moiety of the *N gonorrhoeae* endotoxin The complete endotoxin is a lipopolysaccharide protein complex Chemical analyses now in progress have shown that the aqueous ether endotoxin contains a dominant protein moiety *Homma et al* (5 6) working with the endotoxin from autolyzed *Pseudomonas aeruginosa* found antigenic determinants both in the protein and in the polysaccharide moieties of the complex Determinant *b* may in a similar manner belong to the protein moiety of the gonococcal endotoxin However so far no proof has been provided that *a* and *b* belong to the same molecule Since both determinants were sedimented by high speed centrifugation the following possibilities remain 1) *a* and *b* may represent different determinants of one and the same macromolecular complex 2) They may be antigenic determinants of seperate macromolecular complexes The aqueous ether endotoxin has been selected for further investigations which are chiefly concerned with the relationship between *a* and *b* and the chemical composition and serological properties of the preparation

SUMMARY

Endotoxin was prepared from a strain of *N gonorrhoeae* by extraction with aqueous ether phenol water trichloroacetic acid alkali or by heating The preparations formed opalescent solutions in water and the endotoxin was sedimented by high speed centrifugation All the preparations except the alkali extract provoked a positive epinephrine skin test in rabbits Haemagglutination inhibition and absorption experiments revealed two antigenic determinants Phenol water endotoxin contained only one of these while the other preparations contained both Periodate oxidation and digestions with proteolytic enzymes indicated that one determinant is a polysaccharide and the other a protein The possibility that both determinants belong to the endotoxin of *N gonorrhoeae* is discussed

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THYROGLOBULIN ANTIBODIES IN PATIENTS WITH A POSITIVE DIRECT COOMBS' TEST

By

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Received 14 xii 67

In 1959 *Wasastjerna* (7) described a patient with both haemolytic anaemia and Hashimoto s thyroiditis and two years later *White et al* (10) reported another patient in whom these diseases co existed In 1964 *Shulman et al* (5) described a patient with Hashimoto s thyroiditis haemolytic anaemia and false positive reaction to the tests for syphilis In these three cases the haemolytic anaemia was of the auto immune type with a positive direct Coombs test It therefore seemed of interest to investigate whether sera from patients with a positive Coombs test have a higher incidence of antibodies to thyroglobulin than sera from a normal control series

MATERIAL AND METHODS

All blood specimens which were received in this department during a defined period with a request for a direct Coombs test (with the exception of blood specimens from newborn) and which were positive were examined for their content of both thyroglobulin antibodies and red cell antibodies in the serum. Blood specimens from a total of 54 patients with positive direct Coombs test were investigated

Direct Coombs Test

In this test the thoroughly washed red cells suspended in saline to a 5 per cent suspension were mixed on a glass tile with two different antihuman globulin sera one from a goat and one from a rabbit The rabbit antihuman globulin serum was used in two dilutions 1 100 for demonstration of sensitization with gamma globulin and 1 4 for the demonstration of sensitization with globulin of the non gamma type The test was read after 5 minutes and the strength of the reaction recorded

Investigation of Sera for Blood Group Antibodies

The sera were tested at 37 C with a panel of red cells from selected persons using

1) red cells suspended in saline and followed by an indirect Coombs test
2) red cells suspended in a 30 per cent bovine albumin solution
3) trypsinized red cells suspended in saline

The sera were tested for "cold auto antibodies at 20 C and 4 C with the same panel of red cells suspended in saline When a cold antibody was found a titration in saline was carried out

TABLE
Thyroid Antibodies and Red Cell Antibodies

Case	Diagnosis	Thyroid antibodies			Direct Coombs test		
					Antiglobulin serum from		
		Tanned red cell test	*Eluate*	*Precip test*	goat	rabbit 1 100	rabbit 1 4
NPH	Lymph leucaemia	2500	—	—	++	++	++
LGH	Hypersedimentation	2500	—	—	—	—	++
LKL	Pernic anaemia hypersedimentation	2500	—	—	—	+	++
CHP	Haemol anaemia rheum arthriti	250	—	—	+++	+++	++
PK	Acquired haemol an	250	—	—	+++	+++	+++
AJP	Diss lupus erythem haemol anaemia	250	—	—	—	—	++
VTH	Haemol anaemia	25	—	—	+++	++	+++
VJJ	Haemol anaemia	25	—	—	++	++	+++
EA	Diss lupus erythem	5	—	—	++	++	++

Tryp = trypsin Alb = albumin Sal = saline

Investigation of Eluate from the Red Cells

Eluates from the red cells of the patients prepared by the method of Landsteiner were tested for red cell antibodies by the indirect Coombs technique and were examined for thyroid antibodies by the technique described below

Investigation of Sera and Eluates for Thyroglobulin Antibodies

Precipitating thyroglobulin antibodies were studied by double diffusion in agar gel using a modified Ouchterlony technique (4) In the study of thyroglobulin antibodies with tanned red cell (TRC) test the following method was used Sheep blood cells were formalized by the Weinbach method (8 9) and subsequently treated with tannin and sensitized with thyroglobulin by the method of *Boyden* (1) Thyroglobulin was prepared by the method of *Derrien et al* (2) Sera and eluates were titrated and studied against the sensitized blood cells in Perspex trays by the method of *Fulthorpe et al* (3)

RESULTS

Nine of the 54 sera from Coombs positive patients reacted positively with thyroglobulin sensitized red cells The titres of the thyroglobulin antibodies ranged from 5 to 2500 in three cases 2500 in three 250 in two 25 and in one case 5 However none of the eluates contained thyroid antibody None of the patients had precipitating antibody against thyroglobulin in the serum It is worthwhile mentioning that precipitating antithyroglobulin is to be expected only in sera with high TRC titres There was no correlation between the strength of the Coombs direct test and the titre level in the TRC test There was similarly no correlation between the TRC test and the serum content of warm or cold red cell antibodies in the serum The clinical diagnoses and serological findings in these nine cases are listed in

1
in Nine Patients with Positive Coombs Test

Red cell antibodies in serum							Red cell antibodies in eluate from red cells	
37			Indir Coombs test	20 Sal	4 Sal	4 Alb	37 Sal	Indir Coombs test
Tryp	Alb	Sal						
++	—	—	+	—	4	4	—	—
—	—	—	—	—	2	2	—	+
not done	—	—	—	—	4	8	—	—
—	—	—	—	—	2	2	—	+
—	—	—	—	—	—	—	—	+
—	—	—	—	++	64	64	—	—
—	—	—	—	—	1	2	—	++
++	+	—	—	—	1	2	—	++
+	—	—	+	—	2	4	—	+

Table 1 The strength of Coombs direct test varied Of the nine patients with thyroglobulin antibodies six showed a strongly positive reaction in all three antihuman globulin sera while three showed the strongest reaction in the rabbit antihuman globulin serum diluted 1 4 indicating that the blood cells had been sensitized by globulin of the non gamma type

Red cell auto antibodies of the warm type were found in the sera of three patients and were demonstrable either by the trypsin technique or by Coombs indirect test Cold antibody of the complete type was found in eight out of the nine patients but only one had an abnormally high titre Using the saline technique at 37 C none of the eluates showed any red cell antibody whereas the indirect Coombs test revealed antibody in six cases

TABLE 2
Tanned Red Cell Test for Thyroid Antibodies (TRC)

	TRC test					
	Reaction		Titres			
	Positive	Negative	5	25	250	2500
54 patients with positive Coombs test	9	45	1	2	3	3
106 normal donors	5	101	2	2	1	0

TABLE 3

TRC Test and the Test for Cold Antibodies in Patient AJP Before and After Absorption

	TRC test for thyroid antibodies Serum dilutions				Cold antibodies Serum dilutions								
	1/5	1/25	1/250	1/2500	Undil	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
Unabsorbed serum (AJP)	++	++	+(+)	—	++++	++++	+++	+++	++	+	+	—	—
Serum absorbed with thyroglobulin	—	—	—	—	++++	++++	++++	++++	+++	++	+	—	—
Serum absorbed with normal O blood cells	++	++	++	—	++	++	+	—	—	—	—	—	—

In cases PN and VJJ a trace of a specific blood group antibody (anti e and anti Kidd) in addition to the non specific ones was found In other cases specificity could not be demonstrated

Of 106 normal blood donors with no personal or family history of thyroid disease 4 7 per cent had a positive TRC test The titre in these cases was quite low while in the Coombs positive cases it was at a higher level as is evident from Table 2

As seen in Table 3 one of the patients (AJP) with positive Coombs test had both a relatively strong cold antibody and a strong thyro globulin antibody This serum was investigated by cross absorption The results are shown in Table 3

It can be seen that if a serum containing "cold antibody is absorbed to such an extent that it is free of antithyroglobulin the cold anti body titre is the same as before the absorption

If the cold antibody is absorbed the TRC titre remains unchanged

DISCUSSION

In the present study of 54 patients with a positive direct Coombs test 16 7 per cent were found to have a positive TRC test whilst the same was true of only 4 7 per cent of sera from 106 healthy donors However it is not only the higher incidence in the Coombs positive cases which is worthy of note but also the fact that the TRC titres in the Coombs positive cases are on a far higher level than those in the con trol series In other words these patients seem to possess a certain tendency to form antibodies against more than one tissue This ob servation supports the assumption that these patients have a derailed production of antibodies leading to the formation of antibodies directed against various tissues

It might be imagined that these antibodies consisted of a protein molecule with the ability to combine with various antigens This is however unlikely in view of the fact that red cell antibody and not thyroid antibody was found in the eluates from the red cells This view is thus not supported by the present investigation as no thyroid antibody was found in the eluates from the red cells On the contrary in the case with both "cold agglutinin and a positive TRC it was possible to separate the two antibodies by absorption

This corresponds to the case described by *Wasastjerna* (7) in which the two types of antibody also appeared to be distinctly separated In his case there was similarly no diminution in the titre of the thyroid antibody when the serum was absorbed with human red cells at 37 and 4 C

The present results also agree with those of *Skanse & Nilsson* (6) in patients with hypergammaglobulinaemia They found a positive TRC test in an average of 13 5 per cent in a group of patients with di semin ated lupus erythematosus other diffuse collagen disea s hepatic

cirrhosis multiple myelomas and macroglobulinaemia These authors interpret a positive TRC test as a 'part of a disease pattern involving the appearance of irregular serum antibodies

SUMMARY

Fifty four patients with a positive direct Coombs test were investigated for both thyroid antibodies and red cell antibodies Thyroglobulin antibodies were found in 16 7 per cent as compared with 4 7 per cent of normal blood donors Moreover antithyroid titre was higher than in the normal subjects In the eluates from the red cells only red cell antibodies but no thyroglobulin antibodies could be demonstrated In one case the cross absorption of cold antibody and thyroglobulin antibody indicated that we are dealing with two different antibodies

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INVESTIGATIONS ON THE ENZYMES AND TOXINS OF STAPHYLOCOCCI

Separation of Lipase from Phosphatase and Spectrophotometric Analysis of the 'Egg Yolk Reaction

By

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Gillespie & Alder (3) described the occurrence of an enzyme in coagu lase positive staphylococci which produced opacity on egg yolk me dium The substrate active in the egg yolk reaction was identified as the low density lipoprotein of egg yolk lipovitellenin (6 8) When studied by the agar plate method (5) the egg yolk reaction was found to be composed of clearing and precipitation the latter being due to formation of a mixture of fatty acids which occurred as insoluble salts (8) The enzyme was thus identified as a lipase

The clearing reaction on the other hand was thought to be due to the formation of a soluble residue after removal of the fatty acids from lipovitellenin by the staphylococcal lipase However the possi bility of further degradation of the vitellenin residue by other enzymes *occurring together with lipase also existed For instance the enzyme* acid phosphatase is known to be closely associated with lipase (2 7) The removal of phosphate groups from vitellenin would render the latter readily soluble in water In order to establish whether the egg yolk reaction was caused by the lipase alone the culture supernatants giving the activity were fractionated The present communication also reports studies on the kinetics of the clearing and precipitating reac tions performed with the aid of a spectrophotometric method

MATERIALS AND METHODS

Strain

Among the several strains examined a clinical strain with the lab ratory number 44 was found to be a high lipa e producer (5) This strain had the phage pattern 6 47 53 at the routine test dilution and thu belonged to group III The bacteri were stored in the lyophilized state and taken up on nutrient agar plates before being seeded into the medium for lipase production

Determination of En yme Activity

Lipase was determined by the egg olk agar plate assay method (5) Te t lu tions were filled in 10 mm holes cut in the gar and the reactions were d after 24 hours incubation at 37 C

Phosphatase activity was determined by the method described by *Inniss & San Clemente* (4) using 0 1 ml of test solution and p nitrophenyl phosphate as the sub strate The liberated p nitrophenol was measured at pH 11 and at 400 mμ using a Bausch & Lomb Spectronic 20 colorimeter

EXPERIMENTAL

Production of Lipase

The bacteria were grown for five days at 37 C in one litre flasks containing 200 ml of Difco brain heart infusion broth The cells were centrifuged at 3000 g for 60 minutes at 4 C and the supernatant was filtered through a Seitz clarification filter The culture supernatant was concentrated by ultrafiltration at 4 C using Berkefeld filters coated with 10 per cent collodion in glacial acetic acid Before ultra filtration 0 02 per cent sodium merthiolate was added to the super natant to inhibit bacterial growth After concentrating to twenty times the original volume the supernatant was centrifuged at 30000 g for 60 minutes at 4 C to remove any remaining cells The supernatant was used as the source of lipase

Separation of Lipase from Phosphatase

During studies to be published elsewhere (9) aiming at separating lipase produced by strain 44 from other constituents of the culture supernatants it was observed that the lipase occurred in the void volume of Sephadex G 100 and G 200 and in the inner volume of Sepha rose 4B The latter gel was therefore used in attempts to separate lipase from phosphatase Similar studies made with other strains grown under different conditions will be reported elsewhere (10)

8 ml of the clear ultrafiltered supernatant was applied on a column of Sepharose 4B with a bed size of 1 4 cm diameter and 165 cm length using 0 05 *M* Tris HCl buffer of pH 7 5 The effluent was collected in 8 ml lots using an automatic fraction collector Measurement of the absorbance at 280 mμ of the fractions showed that two peaks occurred

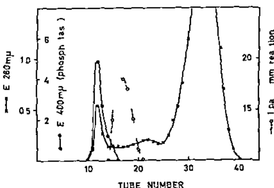


Fig 1

Separation of lipase from phosphatase on Sepharose 4B

(Fig 1) Phosphatase activity was associated predominantly with the first peak representing the void volume and lipase activity occurred in the inner volume close to the first peak The contents of tubes 17 and 18 gave maximal lipase activity

Spectrophotometric Study of the Egg Yolk Reaction

A Unicam spectrophotometer model SP 800 provided with a unit for repeated automatic changing and recording of absorption of four 10 mm light path cells together with their four corresponding blanks was used The cells were maintained at 37 C by circulating water through the cell holder from a thermostated water bath Unless otherwise stated the reaction mixture for these studies consisted of 0 15 ml of egg yolk (12 5 per cent w/v in 10 per cent NaCl adjusted to pH 7 6) and sufficient amount of 0 05 *M Tris HCl* buffer of pH 7 6 to make up the total volume after the addition of the enzyme preparation or other reagents to 3 0 ml The enzyme solution as well as other reagents were added using precision syringes (Hamilton) employed in gas chromatography studies The reaction was recorded at 400 mμ as the solutions were not coloured and the turbidity showed high sensitivity at this wavelength The repetition of recording was set at 5 minute intervals

For the study of the kinetics of the egg yolk reaction the lipase fractionated on the Sepharose 4B column (tubes 16–21 pooled) and free of measurable phosphatase activity was used Uninoculated Difco brain heart infusion broth used as a control showed two effects on the turbidity of egg yolk Up to 0 1 ml of the medium when added to the reaction mixture increased the absorbance due to the brown colour of the medium Higher amounts decreased the initial turbidity of egg yolk due to the presence of salts which solubilized egg yolk The quantity of such salt corresponded to 0 1 M NaCl The solubilizing effect of the medium used as a control was therefore removed on dialysis against 0 05 *M* Tris HCl buffer of pH 7 6 In studies with the purified enzyme preparation the blanks contained all the components of the reaction mixture except for the substrate egg yolk Where the enzyme was replaced as when studying the stability of the turbid egg yolk suspension it was substituted by the buffer

Relationship of enzyme concentration to rate of reaction The reaction mixture containing egg yolk was initially turbid The effect of different amounts of the Sepharose purified enzyme preparation on this turbidity was studied The spectrophotometric recording is shown in Fig 2 In the absence of the enzyme the turbidity of egg yolk remained unaffected for 200 minutes (curve A) In the presence of 80 (curve B) 160 (curve C) and 320 (curve D) microlitres of the enzyme preparation the turbidity of egg yolk diminished at a rate corresponding to the concentration of the enzyme The rates of decrease in absorbance were respectively 0 045 0 10 and 0 20 in absorbance per minute

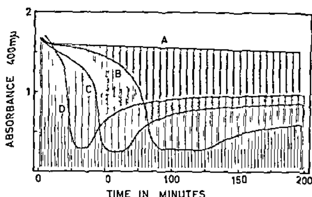


Fig 2

Egg yolk reaction given by lipase fraction from Sepharose 4B A = control without enzyme B C and D contained 80 160 and 320 μl of the enzyme preparation respectively

thus giving a linear relationship to the concentration of the enzyme After reaching a minimum absorbance value of around 0 3 the turbidity increased again With lower concentrations of the enzyme the minimum value was reached after a longer interval than with the higher concentrations 90 minutes with 80 μl enzyme 55 minutes with 160 μl and 30 minutes with 320 μl The rates of increase in absorbance after reaching the minimum values were 0 06 0 12 and 0 18 per minute with enzyme concentrations of 80 160 and 320 μl respectively

The initial fall in turbidity and the secondary increase in absorbance apparently corresponds to the clearing and precipitating reactions noted earlier by the agar plate assay method (5)

Effect of sodium chloride on the egg yolk reaction It is known that egg yolk lipoproteins dissolve in the presence of high concentrations of salt and fall out on removal of the salt by dialysis *Chargaff* (1) used this property to purify lipoproteins It was therefore of interest to

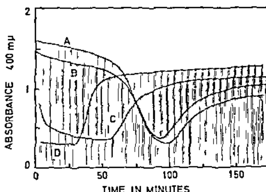


Fig 3

Effect of sodium chloride on the egg yolk reaction 80 μl enzyme A = no NaCl control B C and D contained 0 01 0 1 and 1 0 M NaCl respectively

study the effect of salt concentration on the egg yolk reaction In the presence of 0 01 M NaCl (curve B Fig 3) there was little change in the clearing reaction as compared with the controls without NaCl (curve A) but the precipitation reaction appeared earlier 0 1M NaCl (curve C) diminished the turbidity of egg yolk to such an extent that the clearing reaction could not be studied The precipitation started after about 55 minutes compared with about 100 minutes without NaCl With still higher concentrations of NaCl (1 0M curve D) the turbidity was dissolved and the formation of the precipitate started after about 30 minutes Thus as the salt concentration was increased the time lag for the precipitation reaction was shortened and the rate of precipitation increased This may be due either to increased enzyme activity at high ionic strength or to a salting out effect of sodium chloride on the precipitated fatty acids When assaying the lipase activity by the spectrophotometric method using egg yolk it is obviously important that culture media are checked for the content of salt

Effect of EDTA on the egg yolk reaction Earlier studies (8) had indicated that the precipitate formed during the egg yolk reaction was a mixture of insoluble salts of fatty acids presumably of calcium or magnesium In order to study the reactivity of these ions different amounts of EDTA were used to find the minimum amount needed to inhibit the egg yolk reaction As shown in Fig 4 8 mM EDTA (curve B) increased the rate of the clearing reaction and decreased slightly the initial turbidity of egg yolk compared with the control without EDTA (curve A) The rate of the precipitation reaction was also decreased 16 mM (curve C) and 32 mM (curve D) dissolved the egg yolk and in some experiments a certain increase in the turbidity was noticed when the enzyme preparation was added On the other hand the precipitation reaction was completely inhibited by 16 and 32 mM EDTA

Reversal of EDTA inhibition by calcium and magnesium Using 32 mM EDTA and 160 µl of the enzyme preparation the effect of calcium and

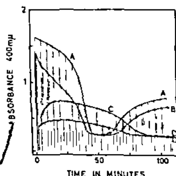


Fig 4

Inhibition of egg yolk reaction by EDTA 160 µl enzyme A control with EDTA B C and D contained 8 16 and 32 mM EDTA

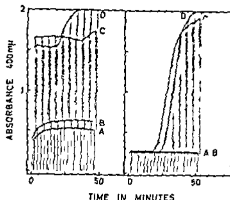


Fig 5

Reversal of EDTA inhibition of egg yolk reaction by calcium chloride Figure to left without NaCl Figure to right with 1M NaCl EDTA concentration 32 mM A B C and D contained 0 0 03 0 3 and 3 mM CaCl

magnesium on the egg yolk reaction was studied The addition of 0 3 and 3 mM calcium chloride (Fig 5 left curves C and D) counteracted the dissolving effect of EDTA on the egg yolk and the turbidity returned No such effect was observed with 0 03 mM calcium chloride (curve B) The clearing reaction was inhibited by 3 mM calcium and the precipitation started after about 20 minutes whereas it took about 40 minutes before the precipitation started if 0 3 mM calcium were used 0 03 mM calcium did not counteract the effect of EDTA during the 50 minutes the reaction was studied

Similar results were obtained with magnesium chloride However magnesium chloride was less effective than calcium as 0 3 mM magnesium (Fig 6 left curve C) did not prevent the clearing reaction

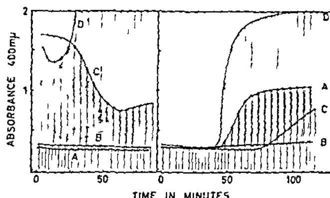


Fig 6

Reversal of EDTA inhibition of egg yolk reaction by magnesium chloride Figure to left without NaCl Figure to right with 1m NaCl EDTA concentration 32 mM A = control without MgCl and in fig to right without EDTA B C and D contained 0 03 0 3 and 3 mM MgCl

Effect of NaCl on reversal of EDTA inhibition by calcium and magnesium In the presence of 1 M NaCl and 32 mM EDTA the turbidity of egg yolk was no longer observable As shown in Fig 5 and 6 (columns to the right) neither calcium nor magnesium up to the concentration of 3 mM studied could reprecipitate the egg yolk lipoproteins The addition of 32 mM EDTA inhibited the secondary precipitation reaction The addition of calcium (Fig 5 right) in increasing amounts showed that 0 3 and 3 mM calcium chloride (curves C and D) reversed the EDTA inhibition both giving similar reaction rates On the other hand the effect of magnesium was less pronounced than that of calcium (Fig 6 right) 3 mM magnesium chloride showed an activity similar to that of 0 3 mM calcium chloride whereas 0 3 mM magnesium showed only a low degree of activity Thus calcium seemed to be about five times as active as magnesium as acyl acceptor in the egg yolk reaction These relative effects of both the actions were more easily observed in the presence than in the absence of 1 M NaCl

DISCUSSION

Results obtained in the present study support the earlier findings with the agar plate assay method (5) where the clearing and precipitation reactions were studied Certain fundamental characteristics of the egg yolk reaction became evident from the spectrophotometric analysis The precipitation reaction is due to the formation of insoluble fatty acid salts of calcium or magnesium (8) Thus the influence of these cations in the egg yolk reaction are explained On the other hand several theories may be put forward to explain the clearing reaction The solubilizing effect of EDTA on lipoproteins and the counteraction of it by calcium together with the observation that the clearing effect due to the action of the enzyme is more pronounced in the absence than in the presence of calcium seems to suggest that the clearing reaction is mainly due to the removal of calcium from the lipoproteins by the enzyme If calcium were bound to the phosphate groups the transfer of calcium to the fatty acids formed would result in solubilization of the residual substrate It is possible that under these conditions the quantity of such fatty acid salts are not sufficient to cause measurable precipitation

The egg yolk reaction is evidently caused by the lipase alone Phosphatase was separated from lipase and found not to influence the egg yolk reaction The solubilization of the lipoproteins by EDTA and reprecipitation by calcium and magnesium offers still another method for the additional purification of the lipoproteins Methods hitherto available for such purification depend upon other characters of the lipoproteins like low density (6 8) and solubility in the presence of high concentrations of salt (1)

SUMMARY

The enzyme lipase produced by a strain of Staphylococcus aureus was separated from phosphatase by gelfiltration on Sepharose 4B. The egg yolk reaction seemed to be caused by the lipase alone.

A spectrophotometric method to be used for the study of the kinetics of the egg yolk reaction is described. Calcium was found to be more active than magnesium as acyl acceptor and the precipitation reaction could be studied more readily in the presence than in the absence of 1 M NaCl. The solubilization of lipoproteins by EDTA and their reprecipitation by calcium or magnesium provides an additional basis for the purification of the egg yolk lipoproteins.

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The National Bacteriological Laboratory Stockholm Sweden

INVESTIGATIONS ON THE ENZYMES AND TOXINS OF STAPHYLOCOCCI

Hydrolysis of Triglycerides and Other Esters by Lipase

By

M O TIRUNARAYANAN and H LUNDBECK

Received 21 xii 67

Lipase activity of staphylococci has been extensively studied since it was discovered by *Eijkman* in 1901 (6) Yet little is known of its relationship to the virulence of the bacteria (7) It has often been demonstrated that the production of lipase by *Staphylococcus aureus* is accompanied by its ability to produce coagulase (8 11 12) a factor generally considered to bear a relationship to the pathogenicity of staphylococci The study of lipase was also of interest from other points of view like for instance its relationship to the haemolysins through formation of lysolecithin (16) as well as its possible role in the production of substances with enterotoxic properties (15) A majority of staphylococci have also been found to give the egg yolk reaction (1 7 13) although the production of lipase is not a common property of pathogenic bacteria (5) These results together with the finding of the frequent occurrence of antilipase antibodies in the sera of humans with staphylococcal infection (14) indicated that the lipase probably plays a role in the ability of staphylococci to multiply within host tissues A similar suggestion was put forward earlier by *Stewart* (20) A detailed study of the lipase of staphylococci was therefore undertaken

Studies on the nature of the reactions on egg yolk (13 21 22) and Tween substrates (23) were reported elsewhere The effect of divalent cations pH and substrate concentration on the lipase activity was established Synthetic triglycerides and other esters were used in the present study to define the characteristics of the enzyme Lipolytic activity on different substrates like egg yolk Tween triglyceride or phenyl ester may be due to a number of similar enzymes occurring together as in vertebrate plasma (3) The investigations by *Stewart* (20) *Torlone & Titoli* (25) and *Vernice* (17) seemed to suggest that a lipase and an esterase were involved These authors observed a partial inhibition of enzyme activity on various substrates by eserine and diethyl p nitrophenyl phosphate On the other hand *Alford Pierce &*

Suggs (2) and *Shah & Wilson* (19) did not obtain any inhibition of the enzyme activity with established esterase inhibitors like atoxyl and tetraaethylpyrophosphate. These latter authors concluded therefore that the reactions were caused by a lipase alone. However none of these workers tried to fractionate the enzyme preparation to study whether the activities were separable. An attempt was therefore made to fractionate the lipase by gelfiltration.

MATERIAL AND METHODS

Strains and culture supernatants. The strains of *Staph. aureus* and the culture supernatants used were the same as described earlier (13).

Determination of lipase activity. A continuous potentiometric titration of the acid produced by the action of the lipase on tributyrin (or other substrates) at various constant pH values was made for the determination of enzyme activity. The equipment used was a Radiometer pH titrator together with the Titrigraph recorder which registered the rate at which NaOH was added automatically to the reaction mixture to maintain the pH at the set constant value. The reaction mixture was stirred by a magnetic stirrer and the vessel was flooded continuously with nitrogen from a gas tube. The reaction vessel was double jacketed and maintained at 37 C by circulating water from a thermostated water bath. The nitrogen gas was first humidified by bubbling through distilled water placed in the water bath to raise the temperature to 37 C before entering the reaction vessel. The reaction mixture for the routine determinations of activity of culture supernatants on tributyrin had the following composition:

0.2 ml of supernatant as source of lipase
0.1 ml of 0.1 M $CaCl_2$ in distilled water
2.6 ml of 0.9 per cent NaCl in distilled water
0.1 ml of 0.1 M tributyrin in acetone

The pH was adjusted to 7.8 before addition of tributyrin. The reagents and the titrant (0.01 N NaOH) were prepared fresh every day. The solutions of tributyrin and other esters used in the present study were tested for shifts of the pH due to hydrolytic products before they were accepted for the experiments. Some batches of the substrates were found to be acidic and had to be discarded.

As controls uninoculated culture media as well as active supernatants which were boiled for 30 minutes were used. These did not produce any acid from the different substrates studied. Some substrates especially phenylacetate gave a low degree of spontaneous hydrolysis. This activity did not exceed 2 per cent of the activity of the highly active enzyme preparations which was however accordingly corrected.

Substrates. The triglycerides and other substrates used in the present study were obtained through the courtesy of Fluka A G, Buchs, St Gallen, Switzerland. The purity of the preparations were triacetin (98 per cent), tripropionin (96 per cent), tributyrin (98 per cent), tricaproin (96 per cent), tricaprin (98 per cent), triolein (97 per cent), amylbutyrate (98 per cent), isobutylbutyrate (97 per cent), methylbutyrate (99 per cent), aethylbutyrate (98 per cent), propylbutyrate (95 per cent), phenylbutyrate (98 per cent). Phenylacetate (99 per cent) was obtained from *Hopkin & Williams* whereas preparations of lecithin, cephalin and sphingomyelin were obtained from Merck, Darmstadt, W. Germany and Light & Co. Ltd., London, England.

EXPERIMENTAL

Preparation of Lipase

A clinical strain with the laboratory number 41 which was found to produce high amounts of lipase as tested by the agar plate assay methods using egg yolk (13) and Tween 60 (23) as the substrates was

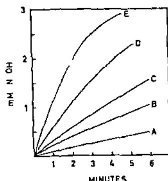


Fig 1

Hydrolysis of tributyrin by staphylococcal lipase E = undiluted enzyme preparation D C B and A = two four eight and sixteen fold dilutions in buffer respectively

used for the preparation of the enzyme The bacteria were grown in one litre bottles containing 500 ml of Difco brain heart infusion broth for 6 days at 37 C The culture was centrifuged at 3000 g for 60 minutes at 4 C and sodium merthiolate was added to the supernatant to a concentration of 0 02 per cent The clear supernatant was concentrated fifty times by ultrafiltration at 4 C using Berkefeld filters coated with 10 per cent collodion The concentrated solution was centrifuged at 30000 g and 4 C for 20 minutes to remove insoluble particles and then adjusted to pH 3 9 and left to stand overnight The sedimented proteins including the lipase were collected by centrifugation at 30000 g as described above redissolved with the aid of a few drops of 1N sodium hydroxide in a minimal volume of 0 05 *I* Tris HCl buffer of pH 7 6 This solution which was about 100 times concentrated was chromatographed on a column of Sephadex G100 The fraction representing the void volume contained all the lipase activity This fraction was used for the study of the kinetics of enzyme activity on various substrates

Relationship between Enzyme Concentration and Reaction Velocity

The effect of different concentrations of the purified enzyme on the rate of acid production from tributyrin as the substrate was studied (Fig 1) Two fold dilutions of the enzyme preparation (from E to A) resulted in a corresponding decrease in the reaction rates as indicated by the titration curves Similar results were obtained with the unfractionated culture supernatants The reaction velocities were linear up to 200 microlitres of the supernatants (Fig 2) Larger amounts gave a sluggish response This was probably due to the buffering action of other proteins produced by the bacteria as well as those found in the culture medium Thus in routine analysis of the culture filtrates or supernatants 0 2 ml was the highest concentration which could be used for enzyme estimation

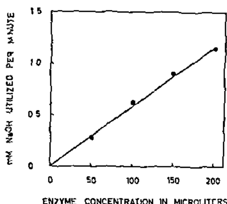


Fig 2

Relationship between enzyme concentration and the rate of hydrolysis of tributyrin

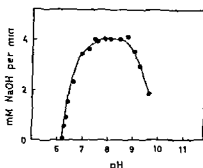


Fig 3

pH activity relationship for the hydrolysis of tributyrin by staphylococcal lipase

Effect of pH Enzyme Activity

Using tributyrin as the substrate and the purified enzyme preparation the rate of hydrolysis at different constant pH values was studied Little activity was found below pH 6 2 (Fig 3) As the pH was raised the activity increased reaching a maximal value around pH 7 5 Further increase in pH did not affect the activity up to a value of pH 8 8 above which the activity decreased Thus the optimum pH for the reaction with tributyrin as the substrate was found to lie between pH 7 5 and 8 8

Effect of Calcium and Magnesium on Enzyme Activity

Preliminary studies with unfractionated culture supernatant showed that the hydrolysis of tributyrin continued in the absence of added calcium or magnesium ions Similar results were obtained also with purified enzyme *Shah & Wilson* (19) supposed that this was due to water functioning as acyl acceptor as the products of hydrolysis were

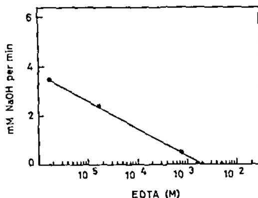
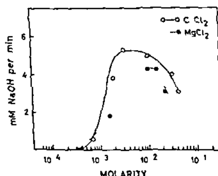


Fig 4
Effect of FDTA on the activit of staphyl c ccal lipase

water soluble In order to ascertain the absence of bound calcium or magnesium the purified enzyme was incubated for five minutes at room temperature with different amounts of I DTA at pH 7 8 There was a linear decrease in activity when the amount of I DTA was increased (Fig 4) 1 6 × 10 ³M EDTA inhibited the reaction completely The FDTA Ca or Mg complex was removed by dialysis against the buffer (0 05 *I* Tris HCl of pH 7 8) and the effect of different amounts of calcium and magnesium chloride on the enzyme activity was studied Using calcium no activity was obtained up to a concentration of 3 3 × 10 M (Fig 5) At higher concentrations the activity increased reaching a maximal value around 2 × 10 M Further increase gradually diminished the initial rate of the reaction especially concentrations above 1 × 10 M Similar activation was noticed with magnesium although magnesium was less effective than calcium Maximal activity



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Rever al cf FDTA inhibition cf lipase by calcium and magnesium l rides

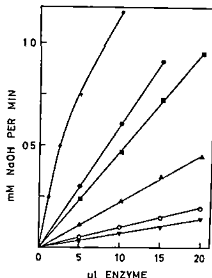


Fig 6

Hydrolysis of triglycerides by staphylococcal lipase

×——× tributyrin ●——● tricaproin ■——■ tripropionin
▲——▲ triacetin ○——○ tricaprin ▼——▼ triolein

with magnesium was observed with concentrations of 1 × 10 M Calcium or magnesium was thus essential for the hydrolysis of tributyrin by lipase and calcium was about three to five times more active than magnesium

Hydrolysis of Different Triglycerides and other Esters

The rates of hydrolysis of the different triglycerides were studied using the purified enzyme preparation a final concentration of 33 mM of the substrates and 3.3 mM calcium chloride The reaction was studied at pH 7.8 and at 37 C The results are shown in Fig 6 The activity with tributyrin was the highest and it decreased in the following order with other triglycerides

tributyrin < tricaproin < tripropionin < triacetin < tricaprin < triolein

Similar studies done with phenylacetate phenylbutyrate and a number of butyric acid esters of alcohols of different chain length showed that the greatest activity was obtained with phenylacetate (Fig 7) The activity decreased in the following order

phenylacetate < phenylbutyrate < isobutylbutyrate < propylbutyrate < amylbutyrate < aethylbutyrate < methylbutyrate

However when the concentration of the substrates was decreased to 3.3 mM the reactivity was in the following order

phenylacetate < phenylbutyrate < amylbutyrate < isobutylbutyrate < propylbutyrate < aethylbutyrate < methylbutyrate (Fig 8)

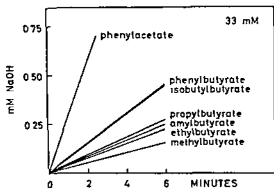


Fig 7

Hydrolysis of phenylacetate and butyrylesters by staphylococcal lipase (substrate concentration 3 3 mM)

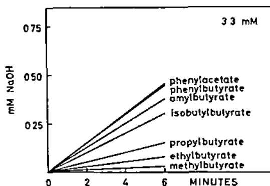
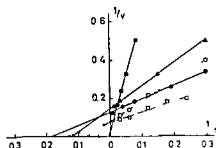


Fig 8

Hydrolysis of phenylacetate and butyrylesters by staphylococcal lipase (substrate concentration 3 3 mM)



F g 9

Lineweaver Burk plots for the evaluati n f M h elis constant for some substrat s of li e

● —— ● tributyrin ▲ —— ▲ aproin □ - - - □ tripropionin
○ - - - ○ triacetin ■ —— ■ t i lein

1/s and 1/v values for tripropion n and tr acetin are as indicated in re Ten times 1/s and half of 1 alues f r tributyrin and tricap i Twent times 1 s and f ve tim s 1/v values for triole i

Thus the activity of phenylacetate was the highest and methyl butyrate the lowest when tested at these two substrate concentrations. Activity on amylbutyrate was lower at 33 mM than at 3.3 mM. Amylbutyrate apparently inhibited the enzyme activity at high substrate concentration. These results demonstrated the importance of using different substrate concentrations when comparing a number of substances before reaching conclusions as to their relative activity. Other factors like the pH as well as concentration of calcium ions should also be considered.

Experiments using cephalin, sphingomyelin and lecithin as one per cent suspensions did not give any activity with the enzyme.

Effect of Substrate Concentration on Reaction Velocity and Evaluation of the Michaelis Constant (Km)

Km values for different ester substrates with varying solubility can only be determined approximately. The values are influenced by the pH, the concentration of cations as well as by the size of the insoluble substrate globules. Nevertheless, a certain characterization of the enzyme can be made under defined experimental conditions. The ester substrates used were dissolved in acetone and 0.1 ml of the acetone solution was added to 2.9 ml of the reaction mixture at pH 7.8. The reaction mixture was composed of 6 µl of the purified enzyme preparation and 3.3 mM calcium chloride and was made up to 2.9 ml with 0.9 per cent sodium chloride solution. Lineweaver-Burk plots relating the reciprocal values of the reaction velocities to the concentration of the substrates using some triglycerides is shown in Fig. 9. Similar results with good linear relationship were obtained with the other esters. From these plots the following values of Km were obtained:

ester compound	*Km (mM)*	*ester compound*	*Km (mM)*
tributyrin	0.55	phenylbutyrate	3.30
tricaproin	0.83	isobutylbutyrate	3.80
tripropionin	7.60	amylbutyrate	9.00
triacetin	13.70	propylbutyrate	16.40
tricaprin	32.00	aethylbutyrate	70.90
triolein	50.00	methylbutyrate	111.00
phenylacetate	16.70		

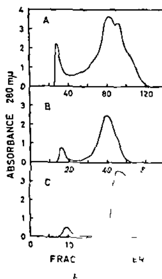
The Km value of 0.55 mM for tributyrin with staphylococcal lipase is in agreement with the value given in the literature for pancreatic lipase which had 0.6 mM for the same substrate (27). On the other hand the aliesterase of liver was found to give a value of 22 mM for methyl butyrate whereas the Km value for staphylococcal lipase was found to be five times higher. The Km values for some substrates for staphylococcal lipase were also reported by *Stewart* (20). He obtained about the same values for tributyrin (0.96 mM), triolein (0.95 mM), phenyl butyrate (0.98 mM) and phenylpropionate (0.98 mM). These values are very different from those obtained in the present study. On the other

hand his results concerning the rate of reaction with tributyrin phenylacetate and triolein corresponded closely to the rates which according to the present authors apply to these substrates The discrepancies between his results and those obtained in the present study can possibly be explained in terms of different enzyme preparations and different techniques used Stewart used an unfractionated aqueous chloroform extract of staphylococci as enzyme preparation the activity of which was studied with the aid of Warburg technique

Experiments to Separate the Different Lipase Activities by Gelfiltration

8 ml of the concentrated culture supernatant without the preliminary acid precipitation step was applied on columns of Sephadex G 100 (2 cm diameter × 144 cm length) Sephadex G 200 (1 9 cm diameter × 149 cm length) and Sepharose 4 B (1 4 cm diameter × 165 cm length) using 0 05 *I* Tris HCl buffer of pH 7 6 10 ml lots of the effluent were collected from columns of Sephadex G 200 and Sepharose 4 B whereas 8 ml portions were collected from the column of Sephadex G 100 using an automatic fraction collector Absorbance at 280 mμ was measured using 10 mm light path cells with a Beckman spectrophotometer model DU The absorbancy values give the separation pattern shown in Fig 10 The first peak on Sephadex G 100 (A) had a high absorption compared with similar peaks from Sephadex G 200 (B) and Sepharose 4 B (C) On all these three gels two major fractions could be separated

The lipolytic activity of the contents of various tubes was measured using egg yolk (13) Tween 60 (23) tributyrin and phenylacetate



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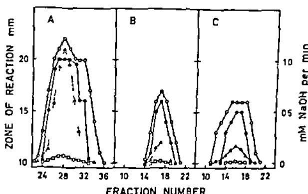


Fig 11

Lipase activity of fractions from gelfiltration on Sephadex G100 (A) Sephadex G200 (B) and Sepharose 4B (C)
Activity on tributyrin Δ---Δ phenylacetate □——□ egg yolk ○——○ and Tween-60 ●——●

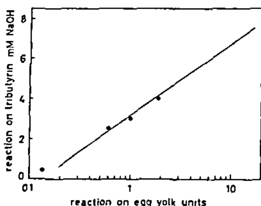


Fig 12

Activity of clinical strains on egg yolk and tributyrin

Lipase activity was located in tubes between 23 and 35 from G 100 gel (Fig 11 A) between 14 and 20 from G 200 gel (B) and between 11 and 20 from Sepharose 4 B gel (C) The first peak on Sephadex G 100 and G 200 contained all the lipase activity whereas the enzyme occurred in the inner volume of Sepharose 4 B The activities on the different substrates appeared closely together indicating that a single enzyme may be involved However other fractionation methods should be used before a definite conclusion can be made

Comparison of the Production of Lipase by Clinical Strains as Assayed with Tributyrin and with Egg Yolk as the Substrates

The relationship between the production of lipase by 400 clinical strains as determined with tributyrin and with egg yolk as the sub

strates was investigated The object of these studies was to find out whether strains producing enzyme splitting tributyrin but not egg yolk or vice versa occurred Such findings would add valuable information concerning the question of identity or non identity of the two activities The results presented schematically in Fig 12 showed that the reactions on egg yolk and tributyrin were fairly well correlated It was also found that strains showing up to 0 2 units on egg yolk agar plates (13) gave no significant activity with tributyrin Similar results were obtained earlier with Tween 60 (23)

DISCUSSION

A certain characteristic feature of the staphylococcal lipase becomes evident from the present studies The enzyme has a high molecular weight and is active in the splitting of a variety of carboxylester bonds *The peak activity is on structures having between four and six carbon* atoms either in the form of acids coupled to glycerol (triglycerides) *or as alcohols coupled to butyric acid (butyrylesters) What possible* deleterious effect such an enzyme can have when liberated within host tissues can hardly be *conjectured On the one hand a number of ester* linkages with Co enzyme A (*e g* butyryl CoA) may be split off resulting in decreased energy output within tissues and muscles On the other hand the liberation of lipase locally around the bacteria will result in an acid pH milieu due to liberation of fatty acids a condition which promotes the biosynthesis of α haemolysin (24)

With a few exceptions mentioned above the results of experiments reported in the present study closely agree with the investigations described by other workers (18 19 20 26) The activity on the various substrates was inhibited by EDTA and counteracted by calcium and magnesium Calcium was found to be more active than magnesium with egg yolk (22) Tween (23) and the triglycerides as the substrates It was not possible to separate the activities on the different substrates by gelfiltration Further the good correlation obtained between the egg yolk reaction and the reactions on Tween 60 (23) as well as tributyrin by the supernatants of 400 clinical strains suggested that the same enzyme was involved

SUMMARY

The activity of staphylococcal lipase against a number of triglycerides and other synthetic esters was studied It was observed that maximal activity was obtained with tributyrin as the substrate A pH optimum between 7 5 and 8 8 and the requirement of a divalent cation such as calcium was established As in other studies with egg yolk and Tween calcium was found to be more effective than magnesium as fatty acid acceptor *Gelfiltration studies showed that the enzyme had a size* corresponding to molecular weights between 500 000 and 1 000 000 and

there was no indication that the lipolytic activities on different substrates were caused by more than one enzyme. Staphylococcal lipase was preferentially active against compounds having between four and six carbon atoms either as fatty acids coupled to glycerol or as alcohols coupled to butyric acid.

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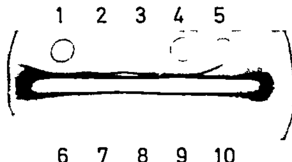


Fig 1

Central well Serum R 374 (bovine) absorbed with human antigen (1H) Cups 1-2-3-4 Bovine antigen (23 22 21 20) Cup 5 Human antigen Cups 6-7 8-9 BCG antigen (16 17 18 19) Cup 10 kansasii antigen (30K)

R 813 Ba (Balnei) immunized like R 700 with Ba (Bal 10)
R 697 A (Avian) immunized like R 700 with A 24
R 699 AI (Avian like) immunized like R 700 with 58 AI
R 829 AI (Avian like) immunized like R 700 with 63 AI
R 709 immunized like R 700 with 52 F (1500)
R 814 immunized like R 700 with 51 Ty (505)
R 736 (Bovine) immunized like R 700 but using killed instead of living culture

R 374 (Bovine) For immunization is u ed a mixture of the following antigens B 20 and B 23 with addition of dialysed and lyophilized ant gen from B 23 B 22 and BCG 17 This antigen mixture is used as an emulgation in an equal quantity of incomplete Freund adjuvans The rabbits were immunized with 0 6 ml of this emulgation This volume was divided in three parts which were injected intra cutaneously into three separate locations of the skin 15 and 45 days later this treatment was repeated Blood samples were taken 24 days after the last injection

R 312 (Battey) immunized like R 374 with a mixture of 68 75 88 and dialysed antigen 88

In the preliminary experiments unheated filtrate of Sauton cultures was used for absorption Equal amounts of antigen and immune sera were mixed and incubated for 3 hours at 37 C and left overnight at 4 whereafter the precipitate was removed by centrifugation

In order to avoid dilution of the serum lyophilized antigens were used in the following experiments One ampoule containing antigen coresponding to 20 ml of the culture filtrate was dissolved in 2 ml of saline For absorption 0.2 ml of this solution was emplo ed per ml of immune serum

For practical purposes we have used Klaus Jensen s modification of *Ouchterlony* s method (5) (see Fig 1)

The absorbed sera were placed in th long central basin After 45 minutes dif fusion the 10 group antigens were placed in the small basins and the reacti ns were recorded after 24 48 and 72 hours In orde to avoid dehydration et filter paper was plac d in the lids of the dishes aft r the first reading

We have also made a thorough t t with the l s i al method Practically the same results were obtained but this meth d p o e l to be less sensitive than the modified one

EXPERIMENTAL RESULTS

Each of the 8 groups of sera were absorb d with the 10 group antigens that we had demonstrated by m ns f the Schultz Dale method

These tests showed in many c ses that it was not sufficient to absorb with one group antigen and it was also evident which combinations had to be used in order to absorb the sera in question

	1H	23B	33Ba	36K	88Bat	24A	58Al	51Ty	52I	84SN	
R 577 H	+	+	+	+	(+)	(+)	(+)	0	0	0	
R 577 H abs 23 B	+	0	0	0	0	0	0	0	0	0	
R 577 H abs ? B	+	0	0	0	0	0	0	0	0	0	
R 577 H abs 33 Ba	+	(+)	0	0	0	0	0	0	0	0	Group I
R 577 H abs 36 K	+	(+)	0	0	0	0	0	0	0	0	
R 577 H abs 88 Bat	+	+	0	0	0	0	0	0	0	0	
R 736 B	+	+	+	+	+	+	+	+	(+)	(+)	
R 736 B abs 1H	0	0	0	0	0	0	0	0	0	0	
R 736 B abs 33 Ba	0	0	0	0	0	0	0	0	0	0	
R 736 B abs 36 K	0	0	0	0	0	0	0	0	0	0	
R 813 33 Ba	+	+	+	+	+	+	+	+	0	0	
R 813 33 Ba abs 1H	0	0	+	0	0	0	0	0	0	0	
R 813 33 Ba abs 23 B	0	0	+	0	0	0	0	0	0	0	Group II
R 813 33 Ba abs 36 K	0	0	+	0	0	0	0	0	0	0	
R 700 49 K	+	+	+	+	+	+	+	+	(+)	0	
R 700 K abs 1H	0	0	0	+	+	0	0	0	0	0	
R 700 K abs 88 Bat	+	+	+	+	0	0	0	0	0	0	Group III
R 700 abs 1H + 88 Bat	0	0	0	+	0	0	0	0	0	0	
R 697 24 A	+	+	+	+	+	+	+	(+)	0	0	
R 697 A abs 58 Al	0	0	0	0	0	+	0	0	0	0	
R 697 A abs 88 Bat	0	0	0	0	0	+	+	0	0	0	Group IV
R 697 A abs 1 H	0	0	0	0	+	+	+	0	0	0	
R 699 58 Al	0	0	0	0	+	+	+	(+)	0	0	
R 699 Al abs 88 Bat	0	0	0	0	0	+	+	+	0	0	
R 699 Al abs 24 A					+	0	+	0	0	0	Group IV O
R 699 Al abs 24 A + 88 Bat	0	0	0	0	0	0	+	0	0	0	
R 814 51 Ty	+	+	+	+	+	+	+	+	(+)	0	
R 814 Ty abs 88 Bat	0	0	+	0	0	0	+	+	0	0	
R 814R 814 Ty abs 33 Ba	0	0	0	0	0	(+)	(+)	+	0	0	Group V
R 814 Ty abs 88 Bat + 33 Ba	0	0	0	0	0	0	0	+	0	0	
R 709 52 I	+	+	+	+	+	0	0	0	+	0	
R 709 52 I abs 1H	0	0	0	0	0	0	0	0	+	0	
R 709 52 I abs 33 Ba	0	0	0	0	0	0	0	0	+	0	Group VI
R 709 52 I abs 88 Bat	0	0	0	0	0	0	0	0	+	0	

The sera and absorbed sera are recorded in the first column The antigen used are listed across the top of the tables H = Human B = Bovine and BCG K = Kansasii Ba = Balnei A = Avian Al = Avian like Bat = Battey SN = *M borstelense* 51 Ty and 52 I
+ indicates a positive precipitation line (+) a weak precipitation line

The final results of the tests with the 8 absorbed sera are recorded in Table 1

From Table 1 it will be seen that it is by means of absorption to produce group specific sera for Groups I–IV

As it is not our intention to make an antigenic analysis of all the mycobacteria we have not included Groups VIII–X in our investigations

Group I If we consider Group I which comprises the human and bovine strains it will be seen that the human serum (R 577) absorbed with several bovine antigens only gives a positive reaction with the human antigens (1–2–3–4–5–6–7–8–9–10–11–12–12–14–15 H) and not with the Bovine antigens (16–17–18–19–20–22–23 B) or with the related strains (Balnei and Kansasii)

Absorption of Bovine sera with human antigens (4 sera were tested including R 736 B) showed that all the antibody had been completely removed from the sera

Using another method for immunization (see R 374) we obtained a quite different result It can be seen from Table 2 that the four Bovine strains 20 21 22 and 23 possess a specific antigen This antigen is not found in the other groups The BCG strains 16 17 18 and 19 either lack this antigen or possess it in quantities too small to be demonstrated by Gel precipitation (see Fig 1)

In the final antigen scheme (see page 457) we have therefore placed the Bovine strains in a separate group

Group II comprises the 4 Balnei strains It is easily distinguished from the remaining groups By absorption of serum R 813 with antigens 1 H 23 B and 36 K all the antibodies are removed with exception of the group specific antibody All 4 Balnei strains show reaction with this absorbed serum

Group III While in the case of Groups I and II it was sufficient to absorb with one antigen in order to obtain a group specific serum we had to absorb with two antigens namely 1 H and 88 Bat for Group III (Kansasii)

This group specific serum showed a positive reaction with 15 Kansasii strains including 53 K and 56 S which with the Schultz Dale method only revealed a small content of the group specific antigen

The results of the tests also indicate that the Kansasii and Battey strains possess a common antigen that cannot be 1 b because it cannot be removed by absorption with the human antigen The fact that two antigens had to be used in order to absorb completely this Kansasii serum indicates the great difficulties that are involved in a complete antigenic analysis of the mycobacteria The result is in fact dependent upon the number of different antibodies to appear upon immunization While the sera used by us for Groups I and II only contained a few common antibodies apart from the group specific antibody serum R 700 contained several common antibodies The result also depends

TABLE 1

	1H	23B	33Ba	36K	88Bat	24Y	58AI	51Ty	52I	94SN	
R 577 H	+	+	+	+	(+)	(+)	(+)	0	0	0	Group I
R 577 H abs 3 H	+	0	0	0	0	0	0	0	0	0	
R 577 H abs 23 H	+	0	0	0	0	0	0	0	0	0	
R 577 H abs 33 Ba	+	(+)	0	0	0	0	0	0	0	0	
R 577 H abs 36 K	+	(+)	0	0	0	0	0	0	0	0	
R 577 H abs 88 Bat	+	+	0	0	0	0	0	0	0	0	
R 736 B	+	+	+	+	+	+	+	+	(+)	(+)	Group II
R 736 B abs 1H	0	0	0	0	0	0	0	0	0	0	
R 736 B abs 33 Ba	0	0	0	0	0	0	0	0	0	0	
R 736 B abs 36 K	0	0	0	0	0	0	0	0	0	0	
R 813 33 Ba	+	+	+	+	+	+	+	+	0	0	Group III
R 813 33 Ba abs 1H	0	0	+	0	0	0	0	0	0	0	
R 813 33 Ba abs 23 B	0	0	+	0	0	0	0	0	0	0	
R 813 33 Ba abs 36 K	0	0	+	0	0	0	0	0	0	0	
R 700 49 K	+	+	+	+	+	+	+	+	(+)	0	Group III
R 700 K abs 1H	0	0	0	+	+	0	0	0	0	0	
R 700 K abs 88 Bat	+	+	+	+	0	0	0	0	0	0	
R 700 abs 1H + 88 Bat	0	0	0	+	0	0	0	0	0	0	
R 697 24 Y	+	+	+	+	+	+	+	(+)	0	0	Group IV
R 697 Y abs 58 AI	0	0	0	0	0	+	0	0	0	0	
R 697 Y abs 88 Bat	0	0	0	0	0	+	+	0	0	0	
R 697 Y abs 1 H	0	0	0	0	+	+	+	0	0	0	
R 699 58 AI	0	0	0	0	+	+	+	(+)	0	0	Group IV O
R 699 AI abs 88 Bat	0	0	0	0	0	+	+	+	0	0	
R 699 AI abs 24 Y					+	0	+	0	0	0	
R 699 AI abs 24 Y + 88 Bat	0	0	0	0	0	0	+	0	0	0	
R 814 51 Ty	+	+	+	+	+	+	+	+	(+)	0	Group V
R 814 Ty abs 88 Bat	0	0	+	0	0	0	+	+	0	0	
R 814R 814 Ty abs 33 Ba	0	0	0	0	0	(+)	(+)	+	0	0	
R 814 Ty abs 88 Bat + 33 Ba	0	0	0	0	0	0	0	+	0	0	
R 709 52 I	+	+	+	+	+	0	0	0	+	0	Group VI
R 709 52 I abs 1H	0	0	0	0	0	0	0	0	+	0	
R 709 52 I abs 33 Ba	0	0	0	0	0	0	0	0	+	0	
R 709 52 I abs 88 Bat	0	0	0	0	0	0	0	0	+	0	

The sera and absorbed sera are [illegible] in the first column. The antigen used are listed across the top of the tables. H = Human, B = Bovine and BC [illegible] K [illegible] Ba [illegible] Y = Avian AI Avian like Bat = Battey SN = W [illegible]steiners 51 Ty and 52 I
+ indicates a positive precipitation line (+ a weak precipitation line

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TABLE 2

	1H	4H	5H	9H	10H	20B	21B	22B	23B	16BCC	17BCC
R 374	+	+	+	+	+	+	+	+	+	+	+
R 374 abs 1 H	0	0	0	0	0	+	+	+	+	0	0
R 374 abs 36 K	+	+	+	+	+	+	+	+	+	(+)	(+)
R 374 abs 88 Bat	+	+	+	+	+	+	+	+	+	+	(+)
R 374 abs 58 Al	+	+	+	+	+	+	+	+	+	+	(+)

	36K	35Ba	58Al	88Bat	51Ts	SN	24A	591	18BCG	19BCC	23B
R 374	+	(+)	(+)	+	+	0	(+)	0	(+)	(+)	+
R 374 abs 1 H	0	0	0	0	0	0	0	0	0	0	+
R 374 abs 36 K	0	0	0	0	0	0	0	0	(+)	(+)	+
R 374 abs 88 Bat	0	0	0	0	0	0	0	0	+	(+)	+
R 374 abs 58 Al	(+)	(+)	0	0	0	0	0	0	(+)	(+)	+

For explanation of Table 2 see Table 1

upon the antigen used for immunization and absorption For purely practical purposes it is however only necessary to produce group specific antibodies as has been done in the present work

Group IV (Avian strains) and Group IV O (Avian like strains) In the present studies we have demonstrated that the Avian like strains possess a specific group antigen which is lacking in the Avian strains This could not be demonstrated with the Schultz Dale method We have consequently altered the antigenic scheme recorded in Report 4

Furthermore our tests showed that the Battey strains possess an antigen common to 24 A and 58 Al and that 58 Al has still another antigen in common with the Battey strains

In the previous works we have mentioned a strain 63 A (B 1208) with the type designation *M avium* With the Schultz Dale method however this was found to be an Avian like strain Upon immunization with this strain we obtained serum R 829 which after absorption with 24 A and 88 Bat only showed reaction with the Avian like strains All the antibody was completely removed from the two Avian like sera R 699 and R 829 when absorbed with the two homologous strains 58 Al and 63 Al

Group VII (Battey) The origin of the Battey strains used by us is listed in Table 3

TABLE 3

Origin of the Battey Strains

No 75	SSC 209	Cultivated from man Statens Seruminstitut Copenhagen
No 68	4004/60	F Fosselt obtained from Kovacs Australia
No 89	7993/60	A F Shilling – – –
No 85	67896/63	J R Griffith – – – –
No 77	19580/60	Pig Strain – – – –
No 78	11601/60	– – – – – –
No 82	11610/60	– – – – – –
No 88	29667/60	– – – – – –

68 89 and 85 are in all probability isolated from man

A good precipitating serum R 312 was obtained by immunizing rabbits with intracutaneous injections of antigen in complete Freund adjuvans The results of absorption experiment with this serum are listed in Table 4 By absorption of serum R 312 with 58 Al or 24 A we obtained a group specific serum which reacted with the Battey strains in our possession It can also be seen from Table 4 that the Battey strains possess an antigen in comm[illegible] [illegible] 4 A 58 Al and 51 Tx

DIS[illegible]SS[illegible]N

By absorption of selected immune [illegible] been practicable to produce group specific sera Thus our pre[illegible] observations from tests with the Schultz Dale method have now [illegible] confirmed

Furthermore we have demonstrated that Group IV O possess a group specific antigen that distinguishes this group from Group IV

TABLE 2

	1H	4H	5H	9H	10H	20B	21B	22B	23B	16BCG	17BCG
R 374	+	+	+	+	+	+	+	+	+	+	+
R 374 abs 1 H	0	0	0	0	0	+	+	+	+	0	0
R 374 abs 36 K	+	+	+	+	+	+	+	+	+	(+)	(+)
R 374 abs 88 Bat	+	+	+	+	+	+	+	+	+	+	(+)
R 374 abs 58 Al	+	+	+	+	+	+	+	+	+	+	(+)
	36K	**35Ba**	**58Al**	**88Bat**	**51Ty**	**SN**	**24A**	**52F**	**18BCG**	**19BCG**	**23B**
R 374	+	(+)	(+)	+	+	0	(+)	0	(+)	(+)	+
R 374 abs 1 H	0	0	0	0	0	0	0	0	0	0	+
R 374 abs 36 K	0	0	0	0	0	0	0	0	(+)	(+)	+
R 374 abs 88 Bat	0	0	0	0	0	0	0	0	+	(+)	+
R 374 abs 58 Al	(+)	(+)	0	0	0	0	0	0	(+)	(+)	+

For explanation of Table 2 see Table 1

and that there is a close antigenic relationship between the Avian and in particular the Avian like strains and the Battey strains In Table 5 we have set up the revised antigenic scheme based on studies previously published as well as on the present work

TABLE 5

Group I	Human	×	–	1	–	a
Group II	Bovine	×	–	2	–	a
	BCG	×	–	(2)	–	(a)
Group III	*M Balnei*	×	–	3	–	a
Group IV	*M Kansasii*	×	–	4	–	a
Group V	*M Avium*	×	–	5	–	b
Group VI	Avian like	×	–	6	–	b
Group VII	*M Battey*	×	–	7	–	b
Group VIII	51 Ty	×	–	8	–	b
Group IX	52 F	×	–	9	–	(c)
Group X	*M borstelense*	(×)	–	10	–	c
Group XI	55 S	×	–	11	–	c
Group XII	54 S	×	–	12		

× indicates the common antigen The figures 1–12 indicate the group specific antigens a b and c indicate the antigens which link the groups together Parentheses signify that the antigen is absent or weak

The antigenic structure of Groups X XI and XII has been determined by means of the Schultz Dale method

The antigenic scheme presented here is far from complete

With regard to Groups X XI and XII no further investigations are being carried out Here we have only included the most important mycobacteria in order to demonstrate the principle of our method for the analysis of antigens which we consider well suited to the practical serological classification of the mycobacteria

It may be feasible to use the same technique for a subdivision of the mycobacteria into types as *Schaefer* (13 14) has done using agglutination

SUMMARY

By employment of absorbed rabbit serum we have been able to produce group specific sera which with *Ouchterlony*s method (12) enabled us to classify the mycobacteria into groups Furthermore using this technique the serological relationship between the different groups has been demonstrated

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AN INDIRECT HAEMAGGLUTINATION TEST FOR SERUM ANTIBODIES AGAINST *MYCOPLASMA PNEUMONIAE* USING FORMALINIZED, TANNED SHEEP ERYTHROCYTES

By

K Lind

Received 23 ii 68

In 1944 *Eaton et al* (11) recovered a causal agent from patients with primary atypical pneumonia (p a p) The agent produced pneumonitis in hamsters and cotton rats and it could be neutralized prior to inoculation by incubation with sera from patients convalescent from p a p (11) This agent has been identified and termed *Mycoplasma pneumoniae (M pneumoniae)* (4 6) and a number of procedures for titration of the specific antibodies have been described

The following tests have been used in various laboratories

1 *The indirect fluorescent antibody test* (FAT) using as antigen either sections of chick embryo lung infected with M *pneumoniae* (28) or colonies of agar grown organisms transferred to slides (4)

2 *Complement fixation (CF) test* was elaborated by *Chanock et al* (5) who used a concentrated phenol treated broth culture of M *pneumoniae* as antigen Later other preparations of the antigen have been employed (21 23 31 13)

3 In 1964 an *indirect haemagglutination (IHA) test* was described by *Dowdle & Robinson* (9) (see below)

4 Two tests are based on inhibition of the metabolism of M *pneumoniae* a) The *tetrazolium reduction inhibition* (TRI) (22) in which colourless tetrazolium is reduced to a red formazan during growth of *M pneumoniae in broth* specific antibody inhibits this reduction b) Another *metabolic inhibition* (MI) test utilizes the decrease in pH caused by fermentation of glucose during growth of M *pneumoniae* in broth the change in pH and its inhibition by antiserum is visualized by the change in colour of phenol red added to the medium (3 16)

5 The *growth inhibition* (GI) *test* (7) is based upon the observations of *Edward & Fitzgerald* (12) that growth of mycoplasma colonies on agar is inhibited by antiserum The test is specific but the sensitivity is too low for diagnostic purpose (33)

This study was aided in part by grants to the Department of Toxoplasmosis and Viral Diseases from *The King Christian X Foundation* and the *U S Public Health Service Research Grant Division* (No AI 01741)

The author is indebted to Miss *Lene Rudbeck* for skilled technical assistance The statistical calculations were kindly performed by *S Olesen Larsen* M A (econ) Biostatistical Department Statens Seruminstitut Copenhagen

6 A direct *haemagglutination inhibition* (HI) *test* with live *M pneumoniae* has been described by *Feldman & Suhs* (14)
7 *Kerr et al* (24) used a *slide agglutination test*
8 *Precipitation techniques* by diffusion in gel are not sufficiently sensitive for the measurement of human antibodies (34 38)
These various techniques have been summarized and the serological results of four different tests have been compared and discussed by *Taylor Robinson et al* (38 39)

In this laboratory the FAT has been used since 1962 (25) It is sensitive and specific but rather time consuming The IHA test was chosen as an alternative test because it is easier to perform and affords almost the same specificity and sensitivity (38 39) The test is based on *Boyden* s findings that tannic acid treated sheep erythrocytes adsorb protein antigen and subsequently agglutinate when mixed with specific antiserum (3) *Dowdle & Robinson* s IHA test for *M pneumoniae* antibodies (9) was modified for titration of antibodies against other human mycoplasmas by *Taylor Robinson* (35)

In the present study the IHA test was applied using formalin treatment of the cells prior to tanning as recommended by *Fliel* (17) *Csizmas (8) and others (29 30) This modification was introduced* because formalinized cells are more stable so that larger batches can be prepared and when tanned and sensitized the cells give more reproducible results Furthermore this procedure could be based on the experience gained in this laboratory with an IHA test for titration of antibodies against *Toxoplasma gondii* (26)

The purpose of this study was to examine the specificity sensitivity and reproducibility of the modified IHA test The results were compared to those obtained by the FAT

MATERIAL AND METHODS

Antigen The Mac strain of *M pneumoniae* was used after 21 to 56 passages on artificial media (25) it was grown in 100 ml volumes of standard broth in Roux bottles until the culture contained 10–10 viable units measured as colony forming units or colour changing units (37) The culture was centrifuged at 40 000 g for 30 minutes and the sediment was suspended in saline and homogenized in a Servall Omnimixer Centrifugation and homogenization were repeated The films of colonies on the inside of the bottles were washed with saline and then removed by shaking the bottles with sterile 3 mm glass beads in saline the suspension of colonies was decanted from the beads and centrifuged disintegrated and washed like the broth culture The materials from broth and film were used either separately or mixed the film representing the major part. After resuspension and addition of penicillin 1000 U/ml the antigenic concentration was about 50 times that of the broth culture This suspension was stored in small amounts in sealed ampoules at —60 C After dilution with seven volumes of phosphate buffered saline (PBS) pH 6 4 the antigen was sonicated at 525 W for one or two hours in a Schoeller apparatus (USIC 750 Data 550 W 19 2 kc)

Treatment of erythrocytes Blood from a sheep selected for this purpose was collected in Alsever s solution and stored from 3 to 10 days at 4 C The erythrocytes were washed 6 times in saline and then formalinized as described by *Weinbach* (40) After washing 6 times in saline a 10 per cent suspension of the cells was made in saline and merthiolate 1 10 000 was added They could then be stored at 4 C for at least 3 months Tannic acid (Merck) was dissolved 1 40 000 in PBS pH 7 2 and incubated at 37 C for 30 minutes with an equal volume of a 3 5 per cent

suspension of formalinized cells in PBS pH 7.2. After centrifugation at 500 g for 10 minutes the tanned cells were washed twice in PBS pH 6.4 and resuspended to 3.5 per cent. They were either used immediately or stored up to a fortnight at 4 C before use.

Sensitization of erythrocytes. The optimal concentration of antigen for sensitization was determined by chessboard titrations of antigen and sera as follows. The sonicated antigen was centrifuged at 40 000 g for 20 minutes, the supernatant was then diluted serially in calibrated tubes and equal volumes of a 3.5 per cent suspension of tanned cells were added. During incubation at 37 C for 30 minutes the tubes were shaken every 5 minutes. After centrifugation at 500 g for 10 minutes the cells were washed once in PBS pH 7.2, then once in the same buffer containing 0.75 per cent heat inactivated (56 C/30 min.) normal rabbit serum (RPBS) (the term normal means that the serum was non reactive in the IHA test). After centrifugation a 1.75 per cent cell suspension was made up in RPBS. Tanned cells treated in the same way but with PBS pH 6.4 instead of antigen were used as controls. Serial two fold or four fold dilutions of a negative serum and a positive serum with known titre were transferred in 0.4 ml volumes to the cups of plastic trays (Prestware). This was done in a chessboard set up where the cells sensitized with various concentrations of antigen were added with one drop (0.04 ml) to each cup. To an identical set up non sensitized cells were added as a control for non specific reactions. Controls were also made for sensitized and non sensitized cells in RPBS without serum. The trays were shaken, protected against evaporation and left at room temperature for 18 hours. The patterns of sedimented cells were read as negative when a distinct ring or button less than 4 mm in diameter was formed. Positive reactions showed cells covering the whole bottom as a film without delineation (+++) or as a film delineated by a ring more than 6 mm in diameter (++). If the endpoint of a two fold serum titration was read as + (i.e. a film bordered by a ring > 4 and < 6 mm) the titre was recorded as 3/4 of the reciprocal value of the particular dilution. The highest concentration of antigen for sensitization of erythrocytes giving the highest specific titre of the positive serum was considered the optimum; the antigen dilution was usually between 1:10 and 1:40.

For some of the experiments tanned cells were sensitized with inactivated horse serum diluted 1:10 in PBS pH 6.4 instead of the sonicated antigen but otherwise treated as described above. The horse serum was from the same batch as that used in the media.

Rabbit Sera

Antisera against *M. pneumoniae* Mac strain (25) were produced in rabbits by antigen given subcutaneously 6 times at intervals of about three weeks; the rabbits were bled a fortnight after the last injection. The Mac strain had been grown in a standard medium (25) modified as described by *Taylor Robinson et al.* (34) using rabbit meat infusion broth with cholesterol instead of ox heart infusion broth and 5 per cent inactivated normal rabbit serum instead of 20 per cent horse serum. The yeast extract was dialysed before the admixture. After gradual adaptation from the standard medium to the rabbit medium the Mac strain had at least four passages in this medium before it was finally grown in a diphasic (agar plus broth) rabbit medium. The culture was then concentrated and washed in saline by alternating centrifugation at 40 000 g and homogenization in a Servall Omnimixer. Equal parts of a concentrated saline suspension of cells and Freund's Complete Adjuvant (Difco) were mixed ... 1:10 000 and 2000 units of penicillin per ml were added. No attempt was made to evaluate the amount of antigen in this suspension which was given in doses of 0.5 ml, the first dose being doubled.

Antisera against the FH strain of *M. pneumoniae* were produced in rabbits using as antigen a culture grown in standard medium and prepared as for the IHA. (The FH strain was isolated in 1954 by Dr *I u* and obtained from Dr *Eaton* through Dr *Chan* ... in 196[illegible]. It was used after the eighth passage in this laboratory.) This antigen was mixed with Freund's complete adjuvant. The animals were given multiple subcutaneous injections of this suspensions twice with an interval of 6 weeks and bled 4 and 6 weeks after the last injection.

Antisera to the other human species of *Mycoplasma* were prepared as for the Mac strain of *M. pneumoniae* with the exception of *M. orale* type 2; antisera to this species were produced in a manner similar to that described for the FH strain of

M pneumoniae The following strains were used *M hominis* type 1 PG 21 *M salivarium* PG 20 *M orale* type 1 Symons *M fermentans* G = FI 9 (25) and *M orale* type 2 20247 which was kindly provided by Dr *Chanock*

The MI test was performed as described by *Taylor Robinson* (37) The FAT and the test for cold haemagglutinins have been described previously (25) A titre of ≧ 160 in the FAT and a cold haemagglutinin titre of ≧ 64 are considered positive

Human Sera

A One hundred and fifty nine sera drawn from voluntary donors in 1965 were kindly provided through the Department of Treponematoses Statens Seruminstitut

B One hundred and ninety six sera were selected at random in several series from 6 497 sera received during 1966 for routine titration of cold haemagglutinins The clinical data of the patients were not recorded the major part of these sera usually originate from patients with respiratory tract infection

C Forty three sera had been selected in the years 1958 to 1965 because of a positive cold haemagglutinin reaction 36 having titres of between 256 and 4000 They were selected from sera received for routine cold haemagglutinin titration as mentioned above

D From the previously reported *M pneumoniae* isolation study (25) 157 sera were available in sufficient quantities for examination by IHA also They originated from 69 of the 77 patients studied in the years 1962 to 1964 and included 38 with primary atypical pneumonia

E During a similar as yet unconcluded isolation study (27) 652 sera were collected in the period March 1966 to June 1967 The study comprised 200 patients with acute febrile respiratory infection (379 sera) and 52 patients without respiratory infection (83 sera) as controls *M pneumoniae* was isolated only from three of the patients with respiratory infection

F From a cold haemagglutinin study of p a p in 1957 and 1958 (26) there remained sufficient amounts of 185 sera for the FAT and IHA test these originated from 94 adult patients who had suffered from acute respiratory infection

G Eighty two pairs of sera were selected because of a rise in titre in complement fixation (CF) tests with viral antigens *viz* 26 pairs with *Influenza A* antigen three with *Influenza B* six with *Influenza C* and 47 with *Adenovirus* antigen They were kindly provided by the Influenza Department Statens Seruminstitut

Another five pairs of sera originated from the Ornithosis Department Statens Seruminstitut where a significant rise in titre against a *Trachoma Inclusion Conjunctivitis* (TRIC) agent had been demonstrated by CF test

H Twenty four sera from patients with infectious mononucleosis showing a strongly positive reaction in the Paul Bunnell test with typical Davidsohn s absorption result were kindly provided by the Streptococcal Department Statens Seruminstitut

J From the same department 17 sera were selected which showed a strongly positive Rose Waaler test

All rabbit and human sera had been stored at —20 C and were inactivated at 56 C for 30 minutes before use

Absorption of sera was performed after inactivation One twentieth volume of packed formalinized (not tanned) cells was added to the serum diluted 1 10 or 1 40 in RPBS After 30 minutes at room temperature the serum was separated by centrifugation When necessary the absorption was repeated

All tests were performed by the same technician

RESULTS

Specificity

The IHA test was investigated by means of sera from patients who had showed a significant rise in titre of antibodies to *M pneumoniae* in the FAT Paired sera were drawn from eight patients who suffered from p a p and from whom *M pneumoniae* had been isolated The rise in titre was with one exception also demonstrated by the IHA test (patient No 9 Table 1)

TABLE 1

Indirect Haemagglutination (IHA) Titres of Antibodies to Mycoplasma pneumoniae Paired Sera Showed a Rise in Titre in the Fluorescent Antibody Test (FAT)

Patient No	Day after onset of illness	FAT	IHA
8	4	< 40	40
	14	1 280	40 000
9	10	< 40	320
	19	160	640
30	8	80	40
	20	2 560	1 280
37	9	160	40
	19	2 560	640
40	8	320	80
	17	1 280	1 280
180	10	40	640
	17	640	2 560
257	5	< 40	160
	15	320	5 000
266	9	< 40	< 40
	21	≧ 2 560	≧ 40 000

M pneumoniae was isolated from all patients

Antisera from rabbits immunized against *M pneumoniae* were tested in the IHA and MI tests All sera gave a positive reaction in both tests while sera taken before immunization did not react Antisera against the FH strain showed a surprisingly high titre in the IHA test as compared with the anti Mac strain sera This difference might be referable to the preparation of the antigens Since the FH strain had been grown in broth supplemented with horse serum the antigen suspension may have contained serum components which resulted in the formation of antibodies to horse serum also The rabbit sera were therefore diluted in PBS with 0 75 per cent horse serum (HPBS) instead of rabbit serum before the addition of sensitized cells This reduced the IHA titre of the anti FH strain serum to the same level as the anti Mac strain serum the titre of which was not changed Furthermore the presence of antibodies against horse serum in the anti FH strain rabbit sera was demonstrated by their strongly positive reactions with cells sensitized with horse serum instead of the sonicated mycoplasma antigen the sera taken before immunization did not react (Table 2) A positive reaction was also obtained when two rabbit antisera against horse serum were tested with these cells It is concluded 1) that the sera from rabbits immunized against the FH strain of *M pneumoniae* contained antibodies to horse serum components in addition to the specific antibodies presumably due to components contaminating the vaccine 2) that the sonicated antigen contained horse serum components which

were coated onto the sheep cells together with the antigen proper giving rise to the fallacious reactions

TABLE 2

Reactions of Rabbit Anti Mycoplasma pneumoniae Sera in the Indirect Haemagglutination Test

Sera: from rabbits immunized against	Sera: diluted in PBS containing 0.75 per cent serum from	Sheep cells sensitized with	Titre
M pneum Ma strain grown in pure rabbit medium	rabbit	*M pneum*	1 280
	horse		1 280
	rabbit	horse serum	< 10
	horse		≦ 10
M pneum FH strain grown in medium with horse serum	rabbit	*M pneum*	40 000
	horse		1 280
	rabbit	horse serum	40 000
	horse		≦ 10

Phosphate buffered saline

Rabbit antisera against the other species of human mycoplasmas were also tested by the IHA and MI tests. All sera from pre immune and immune rabbits reacted negatively in the IHA test with *M pneumoniae* antigen. All antisera inhibited the homologous strains in the MI test.

Eighty seven pairs of human sera were selected because of a rise in titre in CF tests with *Influenza A, B, C, Adenovirus* or TRIC agent antigens (Material and Methods, group G). Eighty five of these pairs were negative with respect to *M pneumoniae* antibodies both in the FAT (titre ≦ 40) and in the IHA test (titre < 40). Two pairs of sera showing a rise in titre of adenovirus antibodies were also reactive in the anti *M pneumoniae* tests. One pair was positive both in the FAT (titre 320 and 160) and in the IHA test (titre 640 and 320), the other pair had FAT titres of 80 and 160 while the IHA titres were both < 40.

The results of FAT and IHA tests on 157 sera from the *M pneumoniae* isolation study (group D) are given in Table 3. The three sera with an IHA titre of 320 and a FAT titre < 80 were from patients whose convalescent sera showed FAT titres of ≧ 160.

Out of 462 sera from the 1966-67 isolation study (group E) 440 had an IHA titre of <40, 14 a titre of 40 and 8 a titre of ≧ 160. Only four sera were positive in the FAT. Three of these were positive also in the IHA test and came from two patients from whom *M pneumoniae* was isolated, the fourth serum was negative in the IHA test and was drawn from a patient without respiratory infection.

TABLE 3

Correlation of Titres in the Indirect Haemagglutination Test and Indirect Fluorescent Antibody Test of 157 Sera from the M pneum Isolation Study (Group D)

IHA Titre	Number of sera								
	78	28	11	12	13	5	7	3	157
≧20 000							1		1
10 000									
5 000							2		2
2 560					1	1	2		4
1 280				2	6	2	2	2	14
640				5	4	1		1	11
320	1	2	1	4	2				10
160			1			1			2
80		4	1						5
40	2		1	1					4
<40	75	22	7						104
FAT titre	<40	40	80	160	320	640	1280	≧2560	

TABLE 4

Correlation of Titres in the Indirect Haemagglutination Test and the Indirect Fluorescent Antibody Test of 185 Sera from Patients with Respiratory Infections in 1957–1958 (Group F)

IHA Titre	Number of sera								
	124	10	6	9	8	6	16	6	18
≧20 000						1	4	5	10
10 000						2	5		7
5 000				1		1	2	1	5
2 560					1		2		3
1 280				1	1	1			3
640			4	3	4		3		14
320			1	2		1			4
160									
80	2	3	1	1					7
40									
<40	122	7		1	2				132
FAT titre	<40	40	80	160	320	640	1280	≧2560	

The correlation of titres in the two tests of 185 sera from the 1957–58 study (group F) is shown in Table 4 The three sera with a positive FAT and an IHA titre of < 40 are from the same patient whose first serum had a titre of < 40 in both tests

One hundred and fifty nine sera from blood donors were examined in both tests (group A) All sera had a FAT titre of ≦ 40 In the IHA test 7 had a titre of 40 in the remainder the titre was < 40

One hundred and ninety six sera were selected at random from the sera received in 1966 for routine titration of cold haemagglutinins

(group B) All but one were negative in the FAT (titre ≦ 40) while 188 had an IHA titre of < 40, 4 of 40 and 4 of 80 the last group including the FAT-positive serum

A series of 43 sera had been selected and stored during the years 1958 to 1965 because of positive cold haemagglutinin reactions (group C) Examinations for *M pneumoniae* antibodies showed that 19 out of 20 sera with a negative FAT had an IHA titre below 40 and one had a titre of 80 The 23 FAT positive sera had titres of ≧ 320 in the IHA test

TABLE 5

Correlation of Titres in the Indirect Haemagglutination Test and the Indirect Fluorescent Antibody Test of the Entire Material

IHA Titre	Number of sera 1222	59	20	30	25	20	29	12	1417
≧20 000						3	5	7	15
10 000				[illegible]		3	5		10
5 000				1	2	2	5	1	11
2 560				1	3	5	7		16
1 280				4	7	3	3	2	19
640		1	4	8	9	2	3	1	28
320	1	2	2	7	2	1		1	16
160	4		1			1			6
80	7	7	[illegible]	2			1	[illegible]	19
40	27	1	1	1					30
<40	1183	48	10	4	2				1247
FAT titre	<40	40	80	160	320	640	1280	≧2560	

The results obtained with all the 1417 human sera examined in both tests are summarized in Table 5 which shows the number of sera grouped according to titre An IHA titre of < 40 was found in 1247 sera six of which had a FAT titre of ≧ 160 An IHA titre of ≧ 160 was found in 121 sera 15 of which had a FAT titre of < 160 These discrepancies together with the significance of slightly elevated titres in both tests will be discussed below

Sensitivity

Table 5 also shows the difference in sensitivity of the two tests The titres of the IHA test were on the average 3 7 times as high as those of the FAT when the calculation was based on sera from the whole material which showed titres of ≧ 160 in both tests

An attempt to evaluate the temporal pattern of the two tests was made by investigating 80 sera collected from 36 patients at various times after the onset of illness only patients developing antibodies to a titre of ≧ 160 in one or both tests were selected The geometric mean titres from each of the first seven weeks are shown graphically in Fig

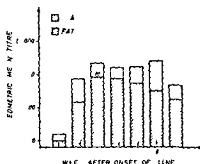


Fig 1

Temporal pattern of serum antibodies to *Mycoplasma pneumoniae* measured by the indirect haemagglutination (IHA) test and the indirect fluorescent antibody test (FAT) The figures indicate number of sera tested by each method

1 In this material the titres of the IHA test were on the average 3 4 times as high as those of the FAT

False Positive Reactions

Patients with infectious mononucleosis generally give a false positive reaction in the IHA test Since the demonstration by *Beer* (1) that horse red cells react in a manner comparable to sheep cells the agglutination of formalinized horse erythrocytes has been devised as a test for heterophilic antibodies in the patients (20) Formalinized sheep erythrocytes react similarly This reaction was demonstrated by 24 sera which showed a high titre in the Paul Bunnell test with a typical Davidsohn s absorption result They were all positive and the reactions with *M pneumoniae* antigen sensitized cells were identical to those obtained with non sensitized cells After absorption of the sera with formalinized sheep cells all reactions were negative (titre < 40) These sera were also negative by the FAT (titre < 40)

A few other sera were encountered which reacted with both sensitized and non sensitized cells generally less strongly with the latter In some instances a specific anti *M pneumoniae* reaction was revealed after absorption with formalinized sheep cells In a few cases however absorptions did not result in the disappearance of the factor(s) reacting with non sensitized cells This phenomenon appeared to occur most commonly in old sera which were heavily contaminated with bacteria generally due to repeated thawing and use

Sera with a titre of < 40 sometimes gave a slightly positive reaction in the 1 10 dilution whether titrated in RPBS or in HPBS and whether absorbed or not The pattern of this was unlike the usual one plus reaction This pattern could also be observed with positive sera showing a prozone phenomenon 1 40 was therefore chosen as the first titration step

A false prozone was often seen with strongly positive sera showing the well known irregular sediment of cells rolling down from the edges of a + + + pattern

The 17 sera with a strongly positive Rose Waaler test were non reactive in the two tests for *M pneumoniae* antibodies

Reproducibility

The reproducibility of the IHA test was evaluated from a total of 101 observations distributed into 18 series In each series the same batch of tanned cells had been sensitized with a constant dilution of the same batch of antigen and tested with the same serum On this basis the standard error was s = 0 128 It means that under the conditions described an error of the titre value by a factor more than 1 8 is to be expected in less than 5 per cent of IHA tests

DISCUSSION

The results lend strong support to the probability that the IHA test measures specific antibodies against *M pneumoniae*

A significant rise in titre of the antibodies was demonstrated in paired sera from patients from whom this organism was isolated The specificity was also illustrated by sera from rabbits immunized with the most common human species of mycoplasma No cross reactions were found between *M pneumoniae* and antigens of other respiratory tract pathogens such as *Influenza A B* and *C* or *Adenovirus* or with a common group antigen of the psittacosis lymphogranuloma venereum trachoma group of agents (TRIC agent) The rare concurrence in the same patient of antibodies against both *M pneumoniae* and *Adenovirus* has been described before this is probably due to sequential infections by these agents (25 18) For practical reasons possible cross reactions with other respiratory tract pathogens were not investigated

Dowdle et al (10) emphasize that a test control designed to detect non specific reactions with horse serum is essential and sera showing such reactions must be absorbed with horse serum sensitized erythrocytes The use of horse serum in the diluent (HPBS) as in the present study seems to be a sufficient and more feasible measure to avoid false positive reactions due to antibodies against horse serum The prevalence of such antibodies in the population has not been investigated For many years until October 1967 about 50 000 doses of antitetanus horse gammaglobulin have been administered annually in this country mainly to adults this means that during the past 10 years about 10 per cent of the adult population has received an injection of horse serum (32) It is the author s impression however that the possibility of the occurrence of antibodies to horse serum in the population giving rise to positive reactions in the IHA test is negligible This is supported by the negative IHA tests of the 159 sera from blood donors from 1963

and by the group of 252 patients in the 1966–67 isolation trial where all 8 IHA positive sera originated from patients with pneumonia

However the results with sera from immunized rabbits emphasize the importance of employing vaccines which contain no antigenic material other than the desired especially in the case of the IHA test

Seventeen sera that were strongly positive in the Rose Waaler test did not react in the IHA test hence the risk that rheumatoid factors might react with the horse serum component coated onto the sheep cells seems to be small To the author s knowledge this reaction has not been described

The reaction of sensitized sheep cells with heterophilic antibodies in sera from patients with infectious mononucleosis indicates that receptors for these antibodies are not blocked by the *M pneumoniae* antigen The fact that this is a false positive reaction is revealed by a positive reaction with non sensitized cells and by its abolition by absorption with these cells

The distribution of the total material according to titre (Table 5) shows that while 1247 out of 1417 sera were non reactive in the IHA test in dilution 1 40 only 30 and 19 had titres of 40 and 80 respectively Fourteen of these 49 sera originated from patients with p a p or from whom *M pneumoniae* had been isolated or from whom another serum showed a titre of ≧ 160 in one of the tests the other 35 sera cannot be accounted for In connection with the high percentage of negative reactions in some of the series discussed below it is assumed that titres of 40 and 80 in the IHA generally indicate low levels of specific antibodies

For routine diagnostic purposes an IHA titre of ≧ 160 or a four fold rise is considered positive For the same reasons this has also been applied to the FAT Titres of 40 and 80 in this test were found in 79 sera (Table 5) 32 of these were from patients with p a p with isolation of *M pneumoniae* or with positive reactions in the IHA test suggesting that these titres are due to low levels of specific antibodies

The results of materials from different periods indicate that the prevalence of *M pneumoniae* infection has varied although an exact evaluation of this is impossible due to different criteria used in the selection of the materials It appears that antibodies to *M pneumoniae* were more commonly registered in this country in the years 1962 to 1964 than in 1965 1966 and the first half of 1967 The high percentage of negative reactions in some of the series of sera from the latter period (groups A B and E) is presumably due to a relatively rapid decline of IHA antibody (10 15) in combination with a low prevalence of the disease In other studies the prevalence of the disease was found to be evenly distributed through the years with a regular seasonal pattern (18 19 10)

The IHA test demonstrates specific antibodies in the second week of illness The material showing the temporal pattern of the two tests

(Fig 1) is too small and subject to too large a standard deviation to show statistically whether the discrepancy in titres is due to different antibodies being measured by the respective tests (2 10 15) For the same reasons one cannot assess the possible interval between the appearance of reactions in the two tests or at what time the reactions disappear

On account of its specificity sensitivity and reproducibility the IHA test is recommended as an aid in the diagnosis of *M pneumoniae* infection

SUMMARY AND CONCLUSION

An indirect haemagglutination test for serum antibodies against *Mycoplasma pneumoniae* using formalinized tanned sheep erythrocytes is described It is specific sensitive and reliable and it detects antibodies in the second week after onset of illness It is a useful aid in the diagnosis of *Mycoplasma pneumoniae* infection

ADDENDUM

In continuation of this study preliminary experiments have justified certain simplifications of the methods

1 The IHA test described may be performed with greater simplicity by the Microtiter system (Flow Laboratories) (35 10) which offers the same sensitivity and reproducibility Serial two fold dilutions of 25 μl serum in HPBS were made in disposable trays (IS MRC-96 Linbro) and 75 μl of a 0 19 per cent cell suspension was added to each cup The trays were sealed with tape left overnight and read with the aid of a mirror

2 Sensitized and control cells were stored in sealed ampoules at —70 C after the addition of dextrose and lactose (38) After storage the cells were thawed *rapidly* at 37 C washed once in HPBS and resuspended in this diluent *There was no haemolysis* and the cells retained their reactivity after *9 months This rationalization* of the sensitization procedure implies a better reproducibility

3 Horse serum was introduced instead of rabbit serum as a stabilizer in the buffer (HPBS) meant for dilutions and suspensions in accordance with the experiences described This generally makes it unnecessary to absorb sera containing antibodies to horse serum

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TRANSACTIONS OF THE PATHOLOGICAL SOCIETY OF NORWAY

Meeting on February 4 1966, Oslo Norway

Kreyberg L LUNG CANCER IN NON SMOKERS

Meeting on March 24 1966, Oslo, Norway

Bet E H (Liege Belgium) SOME IMMUNOLOGICAL ASPECTS OF TUMOUR TRANSPLANTATION

Meeting on June 11, 1966 Bergen Norway

Grov A MODIFICATION OF FUNCTIONAL GROUPS OF PROTEIN ANTIGENS

One method of ascertaining the importance of the functional groups in specific interactions of biologically active proteins is through specific modifications

Protein A (2 3) has been demonstrated in most *Staph aureus* strains and is characterized serologically by giving a specific precipitation line with all normal human sera on agar diffusion Our preparation gives two precipitation lines against rabbit immune serum one of which is identical to the line produced by diffusion against normal human serum In addition the preparation sensitizes tanned sheep erythrocytes to haemagglutination in immune serum as well as in normal human serum

The effect on the serological activities of protein A of chemical modifications of the functional groups carried out by 2 4-dinitro 1 fluorobenzene (DNFB) phenyl iso thiocyanate (PITC) and by diazomethane has been studied (1) Agar gel precipitation and indirect haemagglutination with the reaction products as antigens showed complete loss of serological activity by blocking either of the amino or of the carboxyl groups

Amino as well as carboxyl groups seem to be engaged in all the three serological activities but as indicated by the amount of reagents needed to destroy the individual activities the groups responsible for the specific immune serum line are less accessible or susceptible to modification

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Tonder O SEROLOGICAL INVESTIGATIONS WITH TISSUE SECTIONS

The principle of mixed agglutination is applied to the investigation of antigens and antibodies in tissue Microtome sections of frozen tissue are placed on cover slips The tissue sections can be used with or without fixation before or after incubation with antiserum similar to the immunofluorescent technique Instead of a fluorescent dye erythrocytes are used as the indicator of reactions similar to mixed agglutination with cell suspensions and mixed agglutination with tissue cultures The erythrocytes are added to the tissue in a closed chamber provided by the concavity of a microculture slide and the cover slip

The technique offers the following possibilities for classical serological reactions with tissue sections

1 Simple adsorption of erythrocytes The tissue contains substances with affinity to the erythrocytes *e g* tissue bound antibodies viruses bacteria etc
2 Direct mixed agglutination Antibodies applied to the tissue or to the erythrocytes act as bridge between antigens in the tissue and antigens on the erythrocytes
3 Erythrocytes bound as described in 2 may be lysed by complement and the reaction is transferred to a lytic reaction
4 Indirect mixed agglutination An antiglobulin antibody acts as a bridge between antibodies on the tissue and antibodies on the erythrocytes

The technique is characterized by a very high sensitivity in detecting antigens and antibodies

Lamvik J BLASTOID TRANSFORMATION OF LYMPHOCYTES FROM NEWBORN INFANTS

In mixed cultures containing lymphocytes from two individuals some cells undergo morphological transformation to large blastoid cells which are able to divide This blastoid transformation is thought to be an immunological reaction related to the genetic differences between the donors of the cells

The reactivity of human cord lymphocytes was studied in mixed lymphocyte cultures and compared with the reactivity of adult lymphocytes A significantly higher degree of blastoid transformation was found in mixed cord lymphocyte cultures than in mixed adult lymphocyte cultures In unmixed cord lymphocyte cultures a low degree of blastoid transformation was observed Marked blastoid transformation was observed in cultures of lymphocytes obtained from infants exposed to exchange transfusion No direct evidence of blastoid change in vivo was found after exchange transfusion The high blastoid reactivity of cord lymphocytes may indicate that blastoid transformation is a more primitive immunological reaction than the cellular changes accompanying antibody synthesis

Lexow P TOXOPLASMOSIS WITH NEPHROTIC SYNDROME

In a family in which the parents are unrelated the first two children are healthy but the last four died 16–46 days after birth due to a congenital nephrotic syndrome In three cases the pupils were miotic and one case also had retention type jaundice Three cases came to autopsy and brain tissue is available in two One case had a granulomatous encephalitis with giant cells and calcifications not incompatible with toxoplasmosis Another case had more extensive necrotizing and granulomatous encephalitis with "pseudocysts" diagnosed as toxoplasmosis The mother's blood

showed a marked positive haemagglutination reaction to toxoplasma antibodies (titre 1/160) but negative dye test

The kidneys in the three autopsied cases showed the glomerulopathy with associated tubular dilatation commonly observed in the congenital nephrotic syndrome This lesion is not satisfactorily established as genetically determined

Apart from the purely incidental occurrence of independant congenital nephrotic syndrome and toxoplasma encephalitis these cases indicate that transplacental toxoplasmosis may cause both conditions Vertical transmission of an infectant can give the false appearance of heredity

Staalteki I O PAPILLOMAS IN RECTUM AND COLON

Geertsen J Chr PATHOLOGY IN KOREA

Meeting September 29 1966, Oslo, Norway

SYMPOSIUM ON THE USE OF ELECTRONIC COMPUTERS

Mercoll H INTRODUCTION

Iversen O H GROWTH REGULATION IN MOUSE EPIDERMIS-CARCINOGENESIS BIOLOGICAL ASPECTS

Bjerknes R GROWTH REGULATION IN MOUSE EPIDERMIS-CARCINOGENESIS MODEL SIMULATION

Ruge H SOME PRACTICAL ASPECTS OF AUTOMATED DATA PROCESSING IN MEDICINE

Meeting November 10, 1966 Oslo Norway

Koppang N PORK CONTAINING MERCURY AS A CAUSE FOR POISONING OF DOGS

Although the use of seed treated with mercurial fungicide is not allowed as forage several cases of mercury poisoning have occurred in the post mortem material comprising mostly pigs examined by us poisoning was found to be caused by feeding with dressed seed The organs did not show any distinct pathological anatomical deformations It is therefore possible that pigs fed with dressed seed are slaughtered and sold to consumers

In order to find out whether the comparatively small quantities of mercury which are present in the poisoned meat are able to cause poisoning we started a test on dogs in 1963 As testforage two pigs were used Their livers were known to contain 20 and 30 p p m of mercury respectively The pork was minced and 6 and 9 p p m of mercury respectively in the minced meat were analysed

Two groups were formed In Group No 1 two dogs were fed with two parts of raw meat (6 p p m of mercury) and one part of mush In Group No 2 two dogs were fed with a mash that was cooked of two parts of meat and one part of coarse maize meal Before cooking the minced meat containing 9 p p m of mercury The quantity of mercury in the ready prepared mash was not clinically analysed

During the test period of 66 days the dogs in Group No 1 had about 100 p p m of mercury each On the 47th day one of the dogs in Group No 1 began to show dist n t

symptoms of reduced vision and ataxia Some days later the same symptoms appeared in the other dog in Group No 1 and about one week later also in the dogs of Group No 2

When the dogs were killed on the 66th testing day they all showed distinct cerebral symptoms even though to different degrees Macroscopic examination of the dogs revealed few or no pathologic anatomic symptoms By histological examination of the organs a grained hyalin degeneration was detected in the tubuli contorti of the kidneys and at some places a necrosis of tubules epithelium were present

The greatest deformations were manifest in the nerve system specially in the cerebrum In the meninges there was a slight fibrosis and a moderate infiltration of mononuclear inflammation cells Several of the minor cerebral vessels were thrombosed The larger arteries often showed pathologically thick vessel walls In the cortex cerebri there was a violent decay of nerve cells and a distinct proliferation of astrocytes and mocroglia cells The deformations in some places of the cortex were so distinct as to be characterized as necrosis

The percentage of mercury in the organs of the test dogs varied from 13-25 p p m in the liver from 24-63 in the kidneys while it was 1-14 p p m in the muscular system of the skeleton (All mercury analyses were carried out by Dr A Wülfert chief chemist the Yrkeshygienisk institutt in Oslo)

Reference

Koppang N Forgiftning hos hund ved foring med kvikksølvholdig svinekjøtt Medl blad for Den norske veterinærforening 18 239 245 1966

Haugen O A THE STROM-ZOLLINGER-ELLISON SYNDROME (NON BETA CELL TUMOUR OF PANCREAS)

There has been an increasing interest in the subject of non beta cell tumours of pancreas leading to ulcer formation in the gastrointestinal tract or severe diarrhoea

One case is reported in a 51 years old man who died after 4 years of illness involving dyspepsia During these years he had frequent episodes of gastrointestinal bleeding 2 years prior to death X ray examination showed a gastric ulcer on the greater curvature with possible perforation towards the spleen Scintigraphy of the liver revealed several metastases

At autopsy a non beta cell tumour was found in the tail of the pancreas There was widespread peritoneal metastases and also large metastases to the liver

No ulcer could be tracted but there was a scar on the greater curvature Bilateral I stones were found in the ureters and microscopy showed minor calcifications in the distal tubules in both kidneys The parathyroid glands were macroscopically normal

Froholm L O STRUCTURE AND FUNCTION OF TRANSFER RNA

A review of the known reactions leading to protein biosynthesis was given and the functions of transfer RNA indicated The importance of the template section of one specific aminoacyl transfer RNA molecule out of a number of competing ones was stressed as a step where several control mechanisms might be operating

The recently established primary structures of four transfer RNA molecules (alanyl seryl I & II and tyr syl) from yeast were demonstrated and discussed in relation to specific functional regions

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Meeting on February 16, 1967 Oslo Norway

Marton P MITRAL INSUFFICIENCY (Nord Med 76 1205–1209 1966)

Haugen O A PARATHYREOID PATHOLOGY

Lippestad C & Marton P SINUS ARREST IN PROXIMAL RIGHT CORONARY ARTERY OCCLUSION (Amer Heart J 74 551–556 1967)

Meeting on April 20 1967 Oslo, Norway

Molne K THE ADRENAL CORTEX OF MICE WITH SPONTANEOUS ADRENO CORTICAL LIPID DEPLETION

Arnesen (1956) has described a spontaneous lipid depletion of the adrenal cortex in AKR/O mice and in hybrid lines AC and CS derived from this strain This spontaneous adrenocortical lipid depletion takes place concomitantly with puberty depends upon one recessive gene and is clearly related to the function of the pituitary gland and the gonads The ultrastructure of the adrenal cortical cells in these mice is compatible with a hyperactive state However the plasma corticosterone level in ATCH stimulated males of the CS line is significantly lower than that in control animals

Further studies have been performed to elucidate the function of the lipid depleted adrenal cortex In AC mice the spontaneously occurring lipid depletion mainly takes place between the 30th and 40th day of age This interval coincides with the maturation of the seminiferous epithelium and the degeneration of the adrenal X zone

Hypophysectomy with short time survival leads to adrenocortical lipid repletion This re accumulation of lipids can partially be prevented by large doses of ACTH

The adrenal weight of untreated ACTH stimulated and dexamethasone treated AC males were compared with controls (C57BI) In terms of absolute adrenal weight no difference between the two strains was found but the relative adrenal weight of the AC mice was significantly increased in all groups

Reference

Arnesen K The adrenothymic constitution and susceptibility to leukaemia in mice Acta path microbiol scandinav Suppl 109 1956

Hærem J ACUTE LESIONS IN THE CORONARY ARTERIES IN SUDDEN UNEXPECTED CORONARY HEART DEATH

The purpose of this work was to investigate the frequency of acute lesions in the epicardial coronary a teries in sudden unexpected coronary heart death (SUCHD) and the relationship of the acute lesions to the arteries supplying the sino atrial and the atrioventricular nodes

SUCHD was defined as instantaneous death of persons who had not had any recent symptoms of disease and apparently had been well until the moment of death

After fixation and decalcification the coronary arteries were cut in serial blocks of 2 3 mm each

The material comprised 24 cases 19 males and 5 females of an average of 62 2 (44–85) and 72 6 (64–90) years respectively

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Totally acute lesions in the coronary arteries were found in 19 cases (79 per cent) or in 15 males (79 per cent) and in 4 females (80 per cent) The total number of acute lesions was 35 Among these 22 were judged to be related to the fatal episode In 15 males 40 per cent of the acute lesions consisted of occluding or non occluding platelet fibrin thrombi often related to rupture of intima In the remaining 60 per cent rupture of necrotic atheroschlerotic plaques with embolization of atheromatous debris was found usually with formation of small platelet thrombi at the site of rupture and in one case an occluding haemorrhage in a necrotic atherosclerotic plaque was found

Among the 22 acute lesions which were thought to be related to the fatal episode 16 would possibly have affected the blood supply of the sino atrial node the atrioventricular node or both whereas 6 acute lesions had no such localization This is in accordance with features to be expected on anatomical grounds alone and do not represent any increased frequency of acute lesions related to the sino atrial and atrioventricular nodes

Haugen H F Marton P & Ulstrup J C CYTOMEGALIC VIRUS INFECTION IN MAN (T norske Lægeforen in press)

Meeting on September 28 1967 Oslo Norway

Midtvedt T GERMFREE ANIMALS IN BIOLOGICAL RESEARCH

During the last years a methodology has been developed whereby animals can be maintained and propagated free of a demonstrable microbial flora. These animals are usually referred to as germfree axenic or gnotobiotic animals as distinguished from their conventional parallels Since the animals live in a strictly controlled environment they are suitable for acting as monitors for single strains of micro organisms actually under study In this way it is possible to build up model systems for examination of the symbiotic relationship between the animal and the micro organisms present When their defence mechanisms are reduced these animals are not subjected to infections and they have therefore been used in many kinds of experimental surgery Further with this methodology controlled environments can be provided the latter has been found to of great value both for the protection of Bad risk patients against infections and for isolation of patients with contagious disease

The germfree animals provide without doubt unique experimental advantages in many research programme and the use of such animals will be of help in the solution of man biological problems

Myhre F BLOOD TYPES IN EASTER ISLAND (MAN in press)

Meeting November 2 1967 Oslo Norway

Solheim O I THE EFFECTS OF CONTINUOUS LOCAL IRRADIATION OF BONE MARROW IN RATS

Bone marrow in the femur of rats has been exposed to continuous irradiation by a new technique The effects were found to be different from those of acute exposure and from those reported after continuous whole body irradiation

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S Ijeld R ON THE DISTRIBUTION AND MOVEMENT OF CYTOSOMES AND COLLOID DROPLETS IN THYROID FOLLICLE CELLS

It is well established that TSH administration to hypophysectomized rats or to rats pretreated with thyroid hormons will lead to the appearance of colloid droplets in the follicle cells as well as to an apical movement of the cytosomes The occurrence of colloid droplets being a result of endocytosis can be prevented by the microinjection of endocytic inhibitors into the follicle lumen This procedure will not prevent the apical movement of the cytosomes It thus appears that the endocytosis and the cytosome movement are both effected directly by the TSH and are independent processes as far as the TSH effect is concerned A series of experiments have been planned to test how the two phenomenons are related to other metabolic processes in the thyroid follicle cells This may be done by systemic administration of metabolically active substances to the experimental animal or by the microinjection of suitable inhibitors into the follicle lumen simultaneously with TSH stimulation via the blood Initially the effect of actinomycin D has been tested It has been found that this drug inhibits the formation of colloid droplets on the other hand the movement of cytosomes is not affected This indicates that the endocytosis is governed by a DNA dependant RNA synthesis while the movement of the cytosomes maybe is not It appears that the effect of actinomycin D on the iodine uptake in the thyroid may be explained by this finding

Some general aspects of organized movement of organelles were discussed

Adresen R INFILTRATES OF EOSINOPHILIC GRANULOCYTES IN THE THYROID OF GUINEA PIGS FOLLOWING PASSIVE TRANSFER OF RABBIT ANTI GUINEA PIG THYROID ANTISERUM

Twenty four hours to five days after the intraperitoneal injection of rabbit anti guinea pig thyroid anti erum large numbers of granulocytes probably eosinophilic are found in the guinea pig thyroid (*Godal & Adr sen* 1967)

By light microscopic examination it is demonstrated that the majority of the granulocytes give a positive Dominici staining as well as a positive peroxydase reaction indicating that they are true eosinophilic granulocytes Occasionally non reactive granulocytes are found These may represent neutrophils or degranulated eosinophils

Thirty minutes after the intravenous injection of antiserum eosinophils are found in the wall of small vessels in the thyroid as well as a few in the connective tissue f the gland Such changes are not seen in the controls From 30 minutes to 24 hours after the injections increasing numbers of eosinophils are found in the connective tissue of the glands as well as in the follicle walls and lumina

By electron microscopy the characteristic granules of the eosinophilic granulocytes are demonstrated It is found that numerous granulocytes occupy a position between adjacent f llicle cells in gre tly dilated intercellular spaces which contain a granular material slightly more electron dense than the colloid The junctional complexes are intact however indicating that the e surface spe ializations represent a relative obstacle to the penetrating granulocytes

Numerous granul cytes m re or less devoid of granules are found both in the connective tissue and b tween the epithelial cells Many of these granulocytes contain large vacuoles w th ontents slightly less electron dense than the norm l eosinophilic granule m t x but m re electron dense than the intercellular material This may suggest that the vacu les represent endoc tic vacuoles fu ed with pre existing eosinoph lic g anule

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Asvall J & Hoeg K Alveolar soft part sarcoma (To be published elsewhere)

Roger V and Hoeg K TUMOUR CELLS IN VAGINAL SMEARS IN CLINICALLY LATENT CARCINOMA OF THE OVARY

Two cases of clinically latent carcinoma of the ovaries are reported discovered by identification of tumour cells in vaginal smears

The two women aged 54 and 57 had no symptoms One consulted her doctor because of genital prolaps the other attended a mass screening project In both patients tumour cells typical of adenocarcinoma were present in the vaginal smears. In one case tumour cells were present in 2 out of 3 smears in the other in 2 out of 5 smears In addition psammoma bodies were observed in smears from one of the patients Material from cervix and uterus taken by curretage revealed no signs of malignancy It was therefore deduced that the tumour cells were exfoliated from the ovaries or the Fallopian tubes although any tumour had not been demonstrated by clinical examination One of the patients later developed ascites containing tumour cells of the same type as cells seen in her vaginal smear Both patients were operated upon and both had a cystadenocarcinoma of the ovaries with metastases to the mucosa of the right Fallopian tubes in one of the ones also to the peritoneum

These two cases demonstrate that tumour cells found in the vaginal smear might be of extrauterine origin but that in such cases they are present only sporadically

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University Department of Pathology General Hospital Malmo Sweden

METASTASIS FROM CARCINOMA TO CARCINOMA

By

L Ottosson and T Berge

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Metastasis from one malignant tumour to another was first describe by *Berent* (1902) In that case a squamous epithelial cancer of th lower jaw had metastasized to a renal cancer A search of the subse quent literature up to 1958 revealed 23 cases where one tumour ha metastasized to another All of the tumours were carcinomas Two o them had set up secondaries in a malignant mesenchymal tumou (*Berg* 1955 angiomyoliposarcoma *Posnikoff & Stratford* 1960 malig nant glioma) the others in another carcinoma (Table 1)

Since all the publications reported only single cases it is not possib to say how common such metastasis is In most cases the tumours ha metastasized to a renal cancer We therefore analysed a large autops series for metastasis from one carcinoma to another with speci reference to the kidney

MATERIAL

During a 9 year period 1958–1966 altogether 4 455 carcinomas were demonstrat post mortem at the Department of Pathology Malmo General Hospital The sites a numbers of the primary tumours are given in columns 1 and 2 in Table 2 Column gives the number of metastasizing tumours and column 4 the frequency with whi metastasizing tumours set up secondaries in the kidneys

METHODS

The autopsies were performed in a uniform way during the entire period covered the investigation All the lobes of the lungs the liver the kidneys myocardium a spleen were routinely examined histologically The extent of the examination w otherwise varied according to the nature of the case and was often comprehensi Sections usually many of all macroscopically diagnosed renal tumours were e amined histologically

So called cortical adenoma with diameter of more than 2 cm was regarded renal cancer

All macroscopically and microscopically demonstrated tumour foci remote fr the primary tumour and showing signs of growth were classified as metastases

A metastasis in a carcinoma was accepted as such only when two co existi primary tumours of different histological types were demonstrable and the histo gical picture of the metastases corresponded to that of the other tumour

TABLE 1

Published Cases of Carcinoma that Set up Secondaries in a Co-Existing Carcinoma

Author	Site of metastasizing tumour	Site of recipient carcinoma
Berent (1902)	lower jaw	kidney
Hamman (1927)	thyroid	kidney
Schmorl (1928)	lung	kidney
Walter (1928)	uterus	kidney
Walther (1948)	lung	kidney
	breast	parathyroid
	sinus piriformis	kidney
	stomach	kidney
Jackson & Symmers (1951)	lung	colon
Ortega et al (1951)	chorioid	kidney
Rabson et al (1954)	lung	kidney
	lung	kidney
	prostate	kidney
	prostate	kidney
Walther (1954)	thyroid	stomach
	lung	prostate
	lung	pancreas
Schneider (1955)	prostate	kidney
Dobbing (1958)	lung	kidney
Gore & Barr (1958)	prostate	kidney
	breast	kidney
Boyd (1967)	lung	kidney
	lung	kidney

FINDINGS

Metastasis of one carcinoma to another carcinoma was found in 5 subjects: pulmonary carcinoma in 2, colonic carcinoma in 1, and malignant melanoma in 1 had set up secondaries in renal carcinoma. In addition, in 1 case a pulmonary carcinoma had metastasized to a carcinoid of the small intestine. The cases are summarised in Table 3. The entire series contained 78 cases with renal as well as extrarenal carcinoma. The sites of the extrarenal tumours and the number that had metastasized are given in Table 4.

Twenty-one subjects with metastasizing carcinoma, including 4 with carcinoma of the lung, had co-existing carcinoid.

DISCUSSION

In all of the previous publications it is emphasized that metastasis from one carcinoma to another is extremely rare. Five instances out of about 3,000 metastasizing carcinomas means a frequency of about 1:600. As regards metastasis to renal cancer it was 1:750. This implies that such metastasis is more common than previously supposed. More

TABLE 2

Site and Number of Tumours in the Entire Material Frequency of Renal Metastases

Site	All tumours			Metastasizing tumours			Renal metastases	
	f	m	total	f	m	total	No	%
Prostate	–	809	809	–	207	207	3	1 4
Lung	111	367	478	85	325	410	87	21 2
Stomach	195	246	441	168	207	375	9	2 4
Colon	228	202	430	155	124	279	11	3 9
Breast	372	2	374	347	2	349	25	7 3
Kidney	100	165	265	44	67	111	18	16 2
Pancreas	75	123	198	71	117	188	7	3 7
Liver	54	131	185	34	83	117	14	12 0
Biliary system	115	56	171	99	46	145	4	2 8
Rectum	71	96	167	54	72	126	3	2 4
Ovary	164	–	164	150	–	150	5	3 3
Urinary bladder	38	94	132	22	62	84	11	13 1
Small intestine	43	69	112	14	19	33	0	–
Uterine cervix	96	–	96	82	–	82	7	8 5
Oesophagus	24	51	75	18	40	58	6	10 3
Thyroid	38	18	56	21	14	35	8	22 9
Skin	26	25	51	22	21	43	16	37 2
Uterine body	47	–	47	35	–	35	2	5 7
Unknown	24	18	42	23	18	41	3	7 3
Renal pelvis + ureter	15	9	24	13	8	21	3	14 2
Testis	–	15	15	–	13	13	5	38 5
Appendix	5	8	13	1	3	4	0	–
Vulva	12	–	12	10	–	10	3	30 0
Larynx	0	12	12	0	8	8	2	25 0
Oral cavity	4	8	12	3	6	9	1	11 1
Eye	5	5	10	5	5	10	6	60 0
Maxillary sinus	3	7	10	1	4	5	0	–
Anus	6	3	9	5	2	7	1	14 2
Uterus (unspecified)	9	–	9	8	–	8	0	–
Hypopharynx	1	6	7	1	3	4	0	–
Salivary gland	4	2	6	4	1	5	1	20 0
Epipharynx	1	5	6	1	5	6	0	
Tongue	3	3	6	2	1	3	0	
Adrenal	2	2	4	2	2	4	0	–
Uterine tub	3	–	3	3	–	3	0	
Lip	0	2	2	0	2	2	0	–
Tonsil	0	2	2	0	2	2	0	–
Penis	–	2	2		1	1	0	–
Vagina	2	–	2	1	–	1	0	
Trachea	1	0	1	1	0	1	0	–
Cardia	1	0	1	1	0	1	0	
Sweat gland	0	1	1	0	0	0	0	–
Total	1898	2557	4455	1506	1490	2996	261	8 7

over this figure must be regarded as minimum value Small metastases are difficult to detect at macroscopical examination of a renal cancer with its variegated cut surface and in 3 of the 4 cases the metastases were discovered incidentally at routine histological examination The fourth case was a pigmented secondary from a melanoma that had been seen at gross examination It is therefore probable that secondaries in

TABLE 3

Case Reports

Autopsies	Metastasizing primary tumour	Primary tumour with metastases	Other secondaries from metastasizing primary tumour
1157/63 74 years	lung (adenocarcinoma)	clear cell renal carcinoma	Brain hypophysis liver adrenals small intestine and regional lymph nodes supraclav fossae and right axilla
703/64 77 years	colon (adenocarcinoma)	clear cell renal carcinoma	Liver lungs vertebral bodies adrenals regional lymph nodes liver hilus and left axilla
462/65 40 years	skin (malignant melanoma)	clear cell renal carcinoma	Mesenteric and paraaortal lymph nodes small intestine liver ovaries kidneys adrenals lungs vertebral bodies brain
1543/66 47 years	lung (oat cell bronchial cancer)	clear cell renal carcinoma	Liver spleen mediastinal and supr clav lymph nodes vertebral bodies
868/63 91 years	lung (adenocarcinoma)	carcinoid of ileum (with metastasis in regional lymph node)	Vertebral bodies regional lymph nodes and in supraclav fossa

renal cancers are sometimes missed especially if the histological examination is not very thorough

In 4 out of the present 5 cases and in 18 out of the 23 reported in the literature the metastases were situated in renal carcinomas Various explanations of this predilection of secondaries in cases of renal cancers have been offered One might imagine that renal cancer is particularly common in patients with multiple cancer But judging from our own material and from that of several other investigators (see *Malmio* 1959) it is not

It is well known that renal cancers particularly the clear cell type may grow locally for a long time without infiltration or metastasis It was usually this very type of tumour which *Gore & Barr* (1958) called the dormant type According to *Warburg*s (1956) theories such tumours have not achieved the stage of anaerobic metabolism characteristic of highly malignant tumours and therefore probably provide better soil for the growth of secondaries than do other more malignant tumours with a higher rate of energy metabolism

It might also be assumed that renal cancer with its high lipid and glycogen content may provide suitable environments for invading tumour cells Theories of the specific trophism of renal cancers have been presented (*Berg* 1955) but without any supportive evidence

TABLE 4

Sites of Primary Tumours and the Frequency with which they Set up Secondaries in Subjects with Co Existing Renal Carcinoma

Site	All tumours f	All tumours m	All tumours total	Metastasizing tumours f	Metastasizing tumours m	Metastasizing tumours total
Stomach	5	3	8	5	1	6
Lung	1	3	4	1	3	4
Colon	2	2	4	2	2	4
Pancreas	2	2	4	2	2	4
Breast	7	0	7	4	–	4
Prostate	–	29	29	–	3	3
Biliary system	1	1	2	1	1	2
Ovary	3	–	3	2	–	2
Liver	0	5	5	–	2	2
Trachea	1	0	1	1	–	1
Urinary bladder	0	1	1	–	1	1
Skin	1	1	2	1	0	1
Thyroid	2	0	2	1	–	1
Oesophagus	0	2	2	–	0	0
Small intestine	0	1	1	–	0	0
Rectum	0	1	1	–	0	0
Larynx	0	1	1	–	0	0
Sweat gland	0	1	1	–	0	0
Total	25	53	78	20	15	35

The characteristic histological picture of the clear celled renal cancer contrasts sharply with that of secondaries from a tumour of another histological type (Figs 1 and 2) In other tumours with a less characteristic picture it is not so easy to recognize a metastasis from a co existing tumour This may help to explain why secondaries from other tumours are demonstrated more often in renal tumours

It is clear from Table 3 that as in several cases on record all 4 tumours that had set up *secondaries in renal carcinomas* were very invasive and had affected several organs It appears reasonable to assume that such an abundant shedding of tumour emboli increase the risk of metastasis in a renal carcinoma It has also been claimed that the high frequency of secondaries in renal carcinomas can be explained on a haemodynamic basis About one fourth of the minute volume flows through the kidneys and renal cancers are often very vascular This means that the kidneys receive a large proportion of the emboli shed (*Rabson et al* 1954 *Dobbing* 1958) Compared with this large flow of blood the frequency of renal metastasis is however astonishingly low It is clear from Table 2 that 8 7 per cent of the metastasizing carcinomas set up secondaries in the kidneys Among the 35 subjects with metastasizing extrarenal cancer and co existing renal carcinoma the former type of tumour had metastasized to the latter in 4 (about 11 per cent) This means that the frequency of metastasis to a renal cancer was roughly the same as that to the kidney This is remarkable

TABLE 3
Case Reports

Autopsies	Metastasizing primary tumour	Primary tumour with metastases	Other secondaries from metastasizing primary tumour
1157/63 74 years	lung (adenocarcinoma)	clear cell renal carcinoma	Brain hypophysis liver adrenals small intestine and regional lymph nodes supraclav fossae and right axilla
703/64 77 years	colon (adenocarcinoma)	clear cell renal carcinoma	Liver lungs vertebral bodies adrenals regional lymph nodes liver hilus and left axilla
467/65 40 years	skin (malignant melanoma)	clear cell renal carcinoma	Mesenteric and paraaortal lymph nodes small intestine liver ovaries kidneys adrenals lungs vertebral bodies brain
1543/66 77 years	lung (oat cell bronchial cancer)	clear cell renal carcinoma	Liver spleen mediastinal and supr clav lymph nodes vertebral bodies
868/63 91 years	lung (adenocarcinoma)	carcinoid of ileum (with metastasis in regional lymph node)	Vertebral bodies regional lymph nodes and in supraclav fossa

renal cancers are sometimes missed especially if the histological examination is not very thorough

In 4 out of the present 5 cases and in 18 out of the 23 reported in the literature the metastases were situated in renal carcinomas Various explanations of this predilection of secondaries in cases of renal cancers have been offered One might imagine that renal cancer is particularly common in patients with multiple cancer But judging from our own material and from that of several other investigators (see *Malmio* 1959) it is not

It is well known that renal cancers particularly the clear cell type may grow locally for a long time without infiltration or metastasis It was usually this very type of tumour which *Gore & Barr* (1958) called the dormant type According to *Warburg*s (1956) theories such tumours *have not achieved the stage of anaerobic metabolism character*istic of highly malignant tumours and therefore probably provide better soil for the growth of secondaries than do other more malignant tumours with a higher rate of energy metabolism

It might also be assumed that renal cancer with its high lipid and glycogen content may provide suitable environments for invading tumour cells Theories of the specific trophism of renal cancers have been presented (*Berg* 1955) but without any supportive evidence

metastasizing Only in one of the previously published cases (*Rabson et al* 1954) had the recipient renal cancer metastasized (to the lung) This observation agrees well with the above theory of renal cancer being of a dormant type

The tumour in which a metastasis is most often seen is thus renal carcinoma and the tumour that most often sets up secondaries in another tumour is bronchial carcinoma (9 out of the 23 cases in the literature and 3 out of our 5 cases were situated in the lung)

Jackson & Symmers (1951) who described a case of bronchial carcinoma that metastasized to a colonic carcinoma thought that the metastases in the intestinal mucosa might stimulate the development of cancer This so called induction theory had been put forward as early as in 1924 by *Seecof* and later also by *Dubois Ferrière* (1939) The theory has no experimental basis If the theory were correct one would expect to find cancer induced by metastasis to be most common in organs where the frequency of metastasis is highest

About 2 per cent of all metastasizing carcinomas in the material had set up secondaries in the small intestine (usually from bronchial carcinoma and melanoma) Metastasis to a carcinoid of the small intestine by one of the 21 metastasizing carcinomas in patients with co existing carcinoid can therefore not be regarded as rare As far as metastasis to renal carcinomas is concerned it has already been pointed out that the frequency does not differ from the overall frequency of metastasis to the kidney

The cases described are however too few to warrant safe conclusions as to their frequency

SUMMARY

Five cases of metastasis from one carcinoma to another are reported which brings the total number of known cases of such metastasis up to 28 The phenomenon is not so rare as formerly believed In most cases the tumour had metastasized to a renal carcinoma and the theories put forward to explain this are briefly discussed Metastasis of an extra renal cancer to a renal cancer was found in 1 out of every 750 autopsies that revealed metastasizing carcinoma Four (11 per cent) among the 35 metastasizing tumours in subjects with co existing renal carcinoma had set up secondaries in the latter Of all metastasizing tumours in the series 8 7 per cent had metastasized to the kidney

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ON THE NATURE OF HYALINE MICROTHROMBI

A Light Microscopical Immunofluorescent and Ultrastructural Study

By

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Received 17 xii 67

Hyaline microthrombi (HMT) are round or oval eosinophilic bodies with a diameter varying between 2–3 μ and about 30 μ They are found in small vessels in histological sections from autopsy material

The observation of small hyaline thrombi was first reported by *Welti* (1889) in a study of experimental burns *Apitz* (1938 1942) and *Zinck* (1938) studied the same structures (Fibrinkugelchen) in human material and discussed their possible significance

Unaware of these communications *Skjorten* (1964) reported the presence of HMT in cases of disseminated intravascular coagulation and introduced the term *hyaline microthrombi* The association of HMT with disseminated intravascular coagulation was also stressed by *Hardaway et al* (1965) Subsequently *Skjorten* (1968) has undertaken a quantitative study of the occurrence of HMT in a nonselected autopsy series and found a high incidence of HMT in cases of pneumonia

In the present communication the results of histological immunohistochemical and ultrastructural studies on the nature of HMT will be reported

MATERIALS AND METHODS

This report is based on the study of HMT in 1300 personally performed autopsies and in cases brought to the authors attention among 14100 autopsies performed by the staff of the Department of Pathology Ullevaal Hospital Oslo Norway between July 1 1959 and June 30 1967

Light Microscopical Studies

Autopsies were performed 12–36 hours post mortem. Unless otherwise stated tissue blocks were fixed in 4 per cent formaldehyde and embedded in paraffin Sections were cut at 5 μ The following stains were employed Haematoxylin and eosin (H + E) phosphotungstic acid haematoxylin (PTAH) (*Mallory* 1938) acid picro Mallory (*Lendrum* 1949) Martius scarlet blue (MSB) (*Lendrum et al* 1962) Rosindole reaction (*Glenner* 1957) Lepehne s method for haemoglobin (*Lepehne* 1919) and Turnbull s method for ferrous iron (*Pearse* 1960)

Aided by a grant from *The Norwegian Society for Fighting Cancer*

Immunhistochemical Studies

Pituitaries from four cases with pneumonia were divided in two One half was taken for histological studies and the other half was frozen Cryostat sections were cut at 10 μ and stained with fluorescein conjugated anti sera (*Coons & Kaplan* 1950)

The following antisera purchased from Hyland Laboratories Los Angeles Calif U S A were employed

Fluoresceinated rabbit anti human fibrinogen fluoresceinated rabbit anti human fibrin fluoresceinated rabbit anti human albumin rabbit anti human fibrinogen rabbit anti human fibrin rabbit anti human albumin.

The specificity of all antisera was checked by immune electrophoresis (Statens Institutt for Folkehelse Oslo Norway)

Antifibrinogen and antifibrin showed distinct, identical precipitin lines In addition antifibrinogen showed a faint line which probably corresponded to α macroglobulin Anti albumin showed only one precipitin line different from fibrinogen and fibrin

Controls were as follows

1) Unstained sections

2) Application of non fluoresceinated antisera to sections prior to the application of fluoresceinated antisera

3) Absorbtion of two volumes of fluoresceinated antifibrinogen antiserum with one volume 1 2 per cent purified fibrinogen (Kabi Stockholm Sweden) in 0 3 molar saline Subsequent application to sections The procedure was repeated with fluoresceinated anti fibrin antiserum

4) *Production of pure fibrin clots by addition of 6 NIH units of thrombin in* aqua dest to 0 2 ml 1 2 per cent purified fibrinogen (Kabi Stockholm Sweden) in 0.3 molar saline Application of fluoresceinated anti fibrinogen anti fibrin and anti albumin antisera to frozen sections from these clots

Electron Microscopical Studies

The pituitary from a patient with pneumonia who had particularly many HMT had primarily been fixed in 4 per cent formaldehyde and embedded in paraffin Seven years later it was deparaffinized in xylol postfixed in 1 per cent osmium tetroxide for one hour (*Caulfield* 1957) dehydrated and embedded in Epon 812 (*Luft* 1961) Sections were stained with uranyl acetate and lead citrate (*Reynolds* 1963) and examined in a Zeiss EM 9 electron microscope

RESULTS

Light Microscopical Studies

HMT are round or oval strongly eosinophilic bodies with a diameter which varies between 2–3 μ and about 30 μ The periphery appears dense and homogeneous while the centre of large HMT may contain small vacuoles (Fig 1) At times more irregular less compact HMT may be seen which seem to be composed of densely packed threads (Fig 2) In the H + E stain HMT are easily overlooked because of the difficulty in distinguishing them from other structures The PTAH stain usually gives a deep blue colour similar to that of fibrin Occa

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Figs 1–3

Fig 1 Brain Two HMT distending small vein The larger shows central vacuoles Acid picr Mallory 640 ×

Fig 2 Slightly irregular less compact HMT in vein unattached to vessel wall PTAH 640 ×

Fig 3 Pituitary Two HMT (single arrow) in intimate contact with small quite recent platelet aggregate (double arrow) Acid picro Mallory 640 ×


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sionally however they show a brown or yellowish tinge resembling the colour of fibrinoid (*Pearse* 1960) HMT with less dense thread like *interior* always show a dark blue colour Lendrum s acid picro Mallory *and* MSB stains always give HMT an intensely red colour similar to that of fibrin The Roscindole reaction gives a blue colour similar to the colour of fibrin

Lepehne s stain for haemoglobin was *carried out on sections from* pituitaries and kidneys known to contain many HMT Small HMT were always negative Corresponding to the central vacuoles in large HMT there were occasionally small amounts of brown material with a colour similar to that of erythrocytes in the same sections Only minute po sitive *particles* were seen within vacuoles in HMT Turnbull s stain for haemosiderin was *carried* out on the same sections with negative results

HMT are usually found in *small* vessels veins capillaries and ar terioles They lie singly or in small clusters *apparently floating* freely with the vessels unattached to the vessel wall (Fig 2) Occasionally large HMT seem to plug small vessels (Fig 1) At times HMT *may* be found within ordinary thrombi of different type quite recent platelet aggregates (Fig 3) fibrin thrombi in small vessels (Figs 4 and 5) or mixed thrombi in larger vessels (Fig 6) The association of HMT and conventional thrombi however is an exception rather than the rule — HMT may also be found enclosed *in postmortal* clots in larger veins

Immunofluorescent Studies

There was very little background fluorescence Care had to be taken not to confuse HMT with secretion products lying centrally *in pituitary* acini Only round bodies *lying* clearly within vessels were accepted as HMT These gave a *strong* fluorescence with fluoresceinated anti fibrinogen and anti fibrin antisera (Fig 7) and a distinct but con siderably weaker fluorescence with fluoresceinated anti albumin anti serum Treatment of sections with unconjugated *antisera* prior to the application of the corresponding fluoresceinated *antisera* failed *to* block *fluorescence* which was almost as strong as in *sections treated* only with *fluoresceinated antisera*

Application of fluoresceinated anti fibrinogen and anti fibrin anti sera to frozen sections from clots *produced* by the addition of thrombin

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Figs 4–6

Fig 4 Kidney HMT in afferent arteriol Some lie singly others are incorporated into fibrin thrombus (arrow) PTAH 960 ×

Fig 5 Kidney generalized Shwartzman reaction HMT (arrow) urrounded by compa t fibrin thrombus PTAH 1600 ×

Fig 6 Mixed thrombus *from middle cerebral* artery HMT in thrombus ma s sur rounded by fibrin and platelets H + E 640 ×

to purified fibrinogen gave positive fluorescence but fluoresceinated anti albumin antiserum gave no fluorescence Absorbtion of fluoresceinated anti fibrinogen and anti fibrin antisera with purified fibrinogen prior to the application to sections of pituitaries blocked fluorescence of HMT completely

Electron Microscopical Studies

The tissues were poorly preserved It was difficult to distinguish between cell membranes mitochondria and endoplasmic reticulum even though the cytoplasm contained abundant membraneous material frequently forming large vacuoles Nuclei were fairly well preserved as well as basement membranes and collagen fibrils

Tubular structures lined by flattened cells pressed against a basement membrane were identified as capillaries Within capillaries many HMT with a shape similar to that seen in the light microscope were found (Fig 10) The diameter varied between 2 and 13 μ The HMT never showed any trace of an outer limiting membrane (Fig 9) At low magnification HMT appeared as fairly electron dense homogeneous bodies with well defined peripheral border In the interior there were numerous empty vacuoles with a diameter up to 0 3 μ Neither these vacuoles nor the surrounding structure showed any remnants of cellular organelles At times fibrin bundles with a period of about 220 Å were seen on the surface of or incorporated into the superficial parts of HMT (Fig 8)

At high magnification the body of HMT seemed to be composed of small particles measuring 50–60 × 100 Å These particles frequently formed beaded filaments arranged in a mosaic like pattern In a few areas a period ranging from 160 to 220 Å could be distinguished along the long axis of the filaments

In several small capillaries structures resembling fibrin stars (*Zenker* 1895) were seen (Fig 11) They were composed of slender needles which seemed to radiate from a more compact centre Each needle measured about 0 1–0 2 × 5 μ At higher magnification the stars appeared to be composed of bundles of tightly packed parallel filaments

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Figs 7–9

Fig 7 Pituitary capsule Small vessel with HMT showing strong fluorescence Arrow indicates vessel wall Fluoresceinated anti fibrin antiserum 640 ×

Fig 8 Pituitary Part of HMT On surface fibrin bundle fused with main mass of HMT which consists of irregular mosaic of particles measuring 60 × 100 Å occasionally forming beaded filaments of indetermined length Black lines mesh like interior HMT shows a more irregular periphery without traces of

Fig 9 Pituitary Part of erythrocyte (E) and HMT (T) Erythrocyte shows sharply demarcated periphery Membrane may be distinguished at arrow Loose mesh like interior HMT shows a more irregular periphery without traces of limiting membrane Relatively compact interior 30 000 ×


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E
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W
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No definite periodicity could be distinguished Erythrocytes were seen in small vessels (Figs 9 and 10) They showed a sharply demarcated dense outer zone surrounding a loose mesh of moderate electron density quite different from the interior of HMT (Fig 9) which made identification easy

DISCUSSION

There is good correlation between the results of the present light and fluorescent microscopical studies on the nature of HMT The fibrin staining reactions carried out were consistently positive except for PTAH which occasionally gave a colour similar to fibrinoid These methods however are not chemically specific for fibrin The Rose indole reaction is specific for tryptophan Fibrin contains 3 3 g per cent tryptophan considerably more than most other proteins (*Rauen* 1956) and therefore gives a positive Roseindole reaction

Substances which may give positive reactions with the various fibrin stains are Fibrin coarse collagen fibres elastin myofibrils fibrinoid (*Pearse* 1960) certain macroglobulins (*Jorgensen & Borchgrevink* 1964) and calcified masses Collagen elastin and myofibrils are easily distinguished from fibrin because of morphological differences Calcified masses are basophilic in the H + E stain whereas HMT are strongly eosinophilic Erythrocytes cell nuclei and neuroglia stain blue with PTAH but are negative in the MSB stain

Fibrinoid is no chemically well defined substance (*Montgomery & Muirhead* 1957) It is found interstitially in connective tissue and vessel walls in collagen diseases (*Klemperer* 1960) The socalled fibrinoid found in small vessels in the generalized Shwartzman reaction has been shown to be fibrin by electron microscopy (*Bohle et al* 1959) Material deposited in the intervillous spaces of the placenta was thought to be fibrinoid by some authors *Moe & Jorgensen* (1968) have presented evidence that it is aged fibrin Thus it seems that fibrinoid is a tissue component and is never found in the blood The occurrence of HMT within vessels in the blood therefore makes it unlikely that they are composed of fibrinoid

Our fluorescent antibody studies showed that HMT gave a strong fluorescence with conjugated anti fibrinogen as well as with conjugated anti fibrin It has been demonstrated (*Vazquez & Dixon* 1958) that anti fibrinogen and anti fibrin antisera crossreact in thrombi A posi

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Figs 10–11

Fig 10 Pituitary Large HMT in capillary sh wing many small vacuoles compare Fig 1 In upper right corner part of erythrocyte (E) Capillary wall clearly distinguishable (W) Endothelium fragmented and detached 7 200 ×

Fig 11 Pituitary Fibrin star Slender needles measuring 0 1–0 2 μ × 5 μ radiate from a m se compact centre 13 500 ×

tive fluorescent reaction with these antisera therefore indicate that either fibrinogen or fibrin or both is present

The specificity of our anti fibrinogen and anti fibrin antisera was checked by immune electrophoresis by absorbtion with purified antigen prior to the application to sections and by application to sections from clots produced from purified antigen The results of all these tests indicate a high degree of specificity The failure of unconjugated antisera to block subsequent binding of fluoresceinated antisera is possibly caused by the very strong fibrinogen/fibrin antigens present in HMT It does not invalidate the other specificity tests

Conjugated anti albumin antiserum gave a fluorescent reaction with HMT which was somewhat weaker than the reaction given by anti fibrinogen/anti fibrin but still quite distinct *Gillin & Craig* (1957) showed that a certain albumin content was necessary in *in vitro* produced thrombi in order to obtain positive fibrin staining reactions *Moe* (1968) has shown that application of fluoresceinated anti albumin antiserum to sections from thrombi gives a positive fluorescent reaction particularly at the surface

Ultrastructural studies of fibrin thrombi show an orderly pattern of parallel filaments with a period of 230 Å (*Kay & Cuddingham* 1967) to 250 Å (*Bang* 1965) According to figures given by *Baker* (1958) paraffin embedding of formalin fixed tissues leads to a linear shrinkage of 10 12 per cent Fibrin bundles in our micrographs show a period of 220 Å When the effects of shrinkage are taken into account our measurements fit well with the observation quoted above

In HMT because of the mosaic like pattern of primary filaments a periodicity can only be expected to be seen in scattered areas Only filaments lying exactly in the plane of sectioning can be expected to show the correct period which will correspond to the maximal measurement made 220 Å Again when taking the effects of shrinkage due to primary paraffin embedding into account it seems possible that the filaments which make up HMT may represent fibrin The disorderly organization of filaments in HMT indicates a mode of polymerization different from that of ordinary fibrin This might be due to the presence of fibrinogen fibrin intermediates (*Shainoff & Page* 1962) in HMT Soluble fibrin might contribute to the abnormal fibrin polymerization in HMT *Abildgaard* (1967) has observed the presence of soluble fibrin in the plasma of patients with serious infections Our quantitative studies (*Skjorten* 1968) show a high incidence of HMT in this patient cathegory

Hardaway et al (1963) claimed that HMT are composed of a nucleus of red cells or red cell remnants surrounded by fibrin This claim was based on histological studies using Gridley's Lasan imine Fast Red and Acid Eosin to prove the presence of erythrocytes or fragments thereof They found eosinophilic masses in the centre of small HMT and smaller eosinophilic areas in large HMT Acid stains such as those employed

by *Hardaway et al* (1965) do stain erythrocytes but can neither be considered specific for erythrocytes nor for haemoglobin The only specific methods for haemoglobin are those employing modifications of the peroxydase reaction such as the Lepehne method (*Pearse* 1960) With this method we have obtained completely negative results in small HMT Occasional large HMT however show small fragments of brown staining material in central vacuoles This might represent an artefact *Serck Hanssen* (1965) found sudanophilic material within these vacuoles Our electron micrographs show no remnants of cellular constituents including red cell membranes within HMT Turnbull s stain for haemosiderin was always negative in HMT In keeping with this we failed to find ferritin particles within HMT by electron microscopy

Thus we have found very little evidence to support the theory of *Hardaway et al* (1965) that HMT are formed by fibrin formation on a nucleus of red cells We conclude that HMT are formed by primary aggregation of fibrin

Apitz (1938) stated that HMT were usually formed after death but would not exclude that an occasional HMT might be formed *in vivo* The material studied by us gives evidence that HMT may be formed *in vivo* We have found HMT incorporated in recent platelet thrombi in fibrin thrombi in cases of disseminated intravascular coagulation and in a mixed thrombus occluding the middle cerebral artery The presence of HMT in thrombi is a proof that HMT may be formed *in vivo*

Fibrin formed in tissues and in stagnant blood after death and *in vitro* takes the form of slender needles which frequently fuse to form fibrin stars (*Zenker* 1895 *Apitz* 1942) Ultrastructurally they are composed of tightly packed parallel filaments

On the other hand HMT are globular structures composed of an irregular mosaic of fine beaded filaments The diameter may reach 30 μ The compact structure and globular form of HMT as well as the size suggest that they are formed *in vivo* under the influence of the blood stream Fibrin bundles seen at the periphery of HMT however may have formed agonally or after death of be the result of regular fibrin polymerization *in vivo*

SUMMARY

Hyaline microthrombi (HMT) are round or oval eosinophilic bodies measuring up to 30 μ in diameter They are found in small vessels in sections from autopsy material

Histological and immunhistochemical studies give strong evidence that they are composed of a material derived from fibrinogen with staining properties similar to those of fibrin

Ultrastructurally HMT are composed of densely packed particles

measuring 60 × 100 Å which in favourable areas are seen to form beaded filaments with a period of about 220 Å. When taking into account the effects of the special tissue processing which our material was subjected to, this measurement is compatible with current models for the molecular structure of fibrin.

It is concluded that HMT are composed of fibrin. The disorderly ultrastructural pattern indicates a mode of polymerization different from that of conventional fibrin thrombi.

Histological and ultrastructural findings suggest that HMT are formed *in vivo*.

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A COMPARISON OF THE TUMOURIGENIC EFFECT OF FIVE GRADED DOSES OF 3-METHYLCHOLANTHRENE APPLIED TO THE SKIN OF HAIRLESS MICE AT INTERVALS OF 3 OR 14 DAYS

By

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It has been shown that the carcinogenic effect of repeated applications of a carcinogen to the skin of mice is dependent not only upon the dose but also upon the time intervals between the applications (see for discussion Cramer & Stowell 1943 Vesselinovitch & Gilman 1957 and Vesselinovitch 1958) In a previous publication (Iversen & Iversen 1964) we have reported the tumour yield in the hairless mouse skin after a single and after five repeated applications of exact small graded doses of 3 methylcholanthrene (MCA) in benzene solution As expected five applications gave a higher incidence of papillomas and carcinomas and a shorter latency time than one application of the same concentration of the carcinogen But we do not know how much of this change in tumour appearance was due only to the five times higher total dose and how much depended upon the fact that there is obviously a much longer time of persistence of the carcinogen in the skin after five applications than after one single application The possibility exists that the time that carcinogens are present in the cells is of major importance for the tumour yield

The present work contributes a repetition of some of the previous experiments with exactly the same doses applied however at intervals of 14 days instead of 3 days Its purpose is to investigate the importance of the time factor

MATERIALS AND METHODS

A total of 180 mice of the strain hr/hr was used The animals were housed in plastic cages 10 in each box They were fed the same standard diet as the mice in the previous series The animals were about 15 weeks old at the beginning of the experi-

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ment An equal number of males and females was used The carcinogen applied was 3 methylcholanthrene (Eastman Organic Chemicals) It was dissolved in benzene (Benzolum crystallisabile pro analysis E Merck AG) Solutions of 1/2 1/8 1/16 1/32 1/64 and 1/128 per cent (weight/volume) were prepared by serial dilutions from the first solution shortly before the application One drop of 0 1 ml was administered to the dorsal skin of each animal Five applications were given at intervals of 14 days between each application For each concentration of MCA a group of 30 animals was used The animals were observed every tenth day during an observation period of 19 months Each tumour was recorded and registered as a tumour when present for more than ten days The animals were kept until death or they were killed after 19 months With respect to time the distribution curves of tumour bearing animals were further analysed by means of an electronic computer Whenever possible (*i e* except when precluded by extensive autolysis) a necropsy was made and the tumours examined histologically An attempt was made to differentiate clinically between papillomas and carcinomas as soon as they developed by assessment of degree of infiltration as judged by palpation All lesions registered as carcinomas were eventually histologically verified Infiltration below the *musculus panniculus* was used as the criterium of malignancy Table 2 shows the amount of MCA given to the different groups

TABLE 1

Tumours Appearing on Hairless Mouse Skin up to 19 Months after 5 Consecutive Applications of Different Doses of MCA in Benzene Solution Applied at 3 and 14 Day Intervals Respectively

3 Methylcholanthrene concentration in per cent	Total number of tumours per number of mice alive at appearance of first tumour		Mean number of tumours per mouse alive at appearance of first tumour	
	3 day intervals	14 day intervals	3 day intervals	14 day intervals
1/128		33/28		1.2
1/64	16/21	19/25	0.8	0.8
1/32	41/21	70/30	2.0	2.3
1/16	90/30	137/30	3.0	4.6
1/8	111/25	219/30	4.4	7.3
1/2	140/17	201/28	8.2	7.2

TABLE 2

Final Percentage of Mice with Tumours Appearing on Hairless Mouse Skin up to 19 Months after 5 Consecutive Applications of Different Doses of MCA in Benzene Solution Applied at 3 and 14 Day Intervals Respectively

Dose of 3 Methylcholanthrene			Final percentage of mice with tumours following 5 applications at	
Per cent concentration	Amount per application µg	Total amount µg	3 day intervals	14 day intervals
1/128	8	40		39
1/64	16	80	24	44
1/32	32	160	67	67
1/16	63	315	90	83
1/8	125	625	88	93
1/2	500	2500	82	96

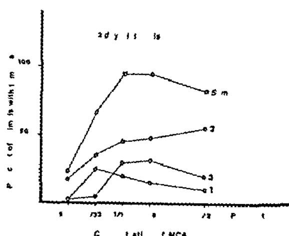


Fig. 1

Percentage of tumour bearing animals after 5 applications of different doses of MCA at 3 days intervals. The upper curve shows the final percentage. The dotted part of the line is theoretical showing when the tumour yield would have reached the 100 per cent level if there had been a straight line correlation between dose and log concentration. The three lower curves give the results for the three different statistical populations (see text p. 51?)

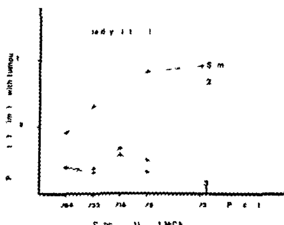


Fig. 2

Percentage of tumour bearing animals after 5 applications of different doses of MCA at 14 days intervals. The upper curve shows the final percentage. The dotted part of the line is theoretical showing when the tumour yield would have reached the 100 per cent level if there had been a straight line correlation between dose and log concentration. The three lower curves give the results for the three different statistical populations (see text p. 51?)

RESULTS

Total Tumours (Papillomas and Carcinomas Considered together)

Table 1 shows that both the total number of tumours and the mean number of tumours per mouse increase with the total dose of the carci

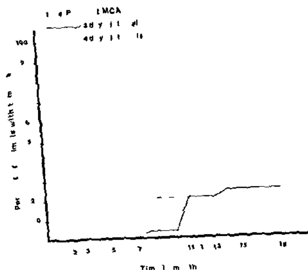


Fig 3

Percentase f tumour bearing animal during the observation period after 5 repeated p intings with 1/64 per cent MCA in benzene at 3 and 14 day intervals respectively

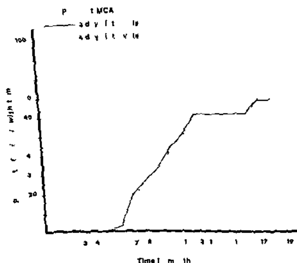


Fig 4

Percentage of tumour bearing animals during the observation period after 5 repeated paintings with 1/32 per cent MCA in benzene at 3 and 14 day intervals respectively

nogen It is seen from Table 2 and from the upper curves of Figs 1 and 2 that in the case of the lowest *concentrations* the final percentage of animals bearing tumours increases with the concentration of MCA almost so as to produce a straight *line on* a semilogarithmic plot This increase is steep when 3 day intervals are used and more slight when

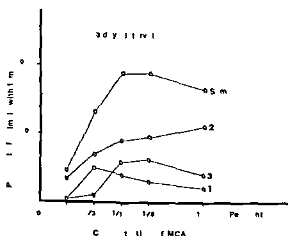


Fig 1

Percentage of tumour bearing animals after 5 applications of different doses of MCA at 3 day intervals The upper curve shows the final percentage The dotted part of the line is theoretical showing when the tumour yield would have reached the 100 per cent level if there had been a straight line correlation between dose and log concentration The three lower curves give the results for the three different statistical populations (see text p 516)

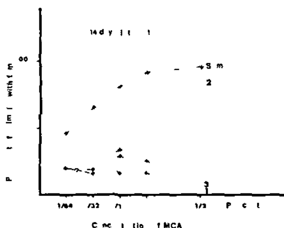


Fig 2

Percentage of tumour bearing animals after 5 applications of different doses of MCA at 14 day intervals The upper curve shows the final percentage The dotted part of the line is theoretical showing when the tumour yield would have reached the 100 per cent level if there had been a straight line correlation between dose and log concentration The three lower curves give the results for the three different statistical populations (see text p 516)

RESULTS

Total Tumours (Papillomas and Carcinomas Considered together)

Table 1 shows that both the total number of tumours and the mean number of tumours per mouse increase with the total dose of the carci

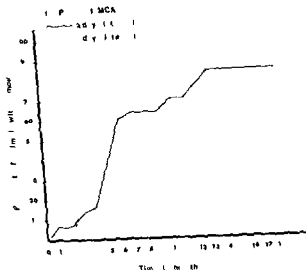


Fig 7

Percentage of tumour bearing animals during the observation period after a repeated paintings with 1/2 per cent MCA in benzene at 3 and 14 day intervals respectively

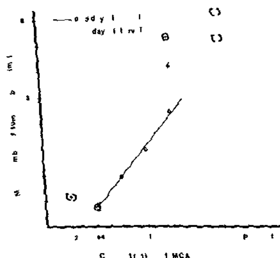


Fig 8

Mean number of tumours per animal after 5 applications of MCA at different concentrations applied at intervals of 3 *and 14 days* respectively Regression lines are drawn for the concentrations between 1/64 *per cent* and 1/8 per cent The values in *brackets* denote the other concentrations. *There is a significant* difference between the two lines (P = 0 05) (One of the animals painted with 1/8 per cent MCA at 14 day intervals developed an *extraordinarily* high number of tumours (40) This animal was therefore excluded *from the calculation* of the regression line *The value* in the circle represents the mean *value if also* this animal had been taken into consideration The statistical difference between the two regression lines would then have been still more significant)

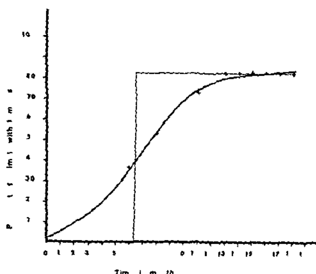


Fig 9

Percentage of tumour bearing animals during the observation period after 5 repeated paintings with 1/10 per cent MCA in benzene at 14 days intervals The + represents the observed values The curve represents a cumulative distribution function calculated on the assumption that this function is the cumulative sum of one normal distribution with a mean value at 6 3 months an amplitude of 85 per cent and the same standard deviation as the observed values The mean value and the amplitude are drawn in the diagram as straight lines

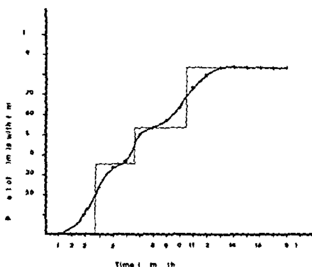


Fig 10

The same observations as in Fig 9 The curve is here the cumulative distribution curve calculated on the assumption that the observed values were the sum of 3 normal distributions with mean values at 3 7 6 6 months and 10 5 months respectively and with the same standard deviation as in the observed values The mean values and the amplitudes are shown in the diagram as straight lines

TABLF 3

Average Time of Occurrence of the Three First Tumours and Mean Latency Time of Three Different Statistical Subpopulations of Tumours Appearing on Hairless Mouse Skin up to 19 Months after 5 Consecutive Applications of Different Doses of MCA in Benzene Solution Applied at 3 and 14 Day Intervals Respectively

3 Methyl cholanthrene concentration in per cent	Average time (in months) of occurrence of three first tumours		Mean latency time (in months) of tumours in first population		Mean latency time (in months) of tumours in second population		Mean latency time (in months) of tumours in third population"	
	Intervals in days		Intervals in days		Intervals in days		Intervals in days	
	3	14	3	14	3	14	3	14
1/64	9 7	3 9	7 5	3 9	10 5	11 3	13 5	16 3
1/32	6 0	3 5	6 6	3 3	10 0	9 6	16 6	15 2
1/16	3 5	3 2	3 6	3 [illegible]	6 4	6 5	14 3	10 5
1/8	4 7	[illegible] 6	4 [illegible]	3 0	7 2	6 1	14 4	11 8
1/2	4 0	2 2	0 9	1 5	4 9	3 4	11 [illegible]	[illegible]

statistical population (see below) is seen from the three lower curves in Figs 1 and 2

Figs 3–7 demonstrate the number of tumour bearing animals in each group during the observation period These curves are compared to the corresponding curves from the earlier series involving three day intervals As zero time for the comparison we have chosen the time of the 3rd application in each group It is seen that in most groups the percentage of tumour bearing animals increases earlier with 14 than with 3 days between the applications It is also seen that on the whole the percentage of mice with tumours is slightly higher in the series that got the carcinogen application with longer intervals (Table 2) but this difference is not constant and not very pronounced (compare also Figs 1 and 2) Assuming a straight line dependency between the logarithm of the percentage concentration of MCA in benzene and the final mean number of tumours per mouse alive at the appearance of the first tumour in the interval from 1/64 to 1/8 per cent regression lines can be drawn as shown in Fig 8 The difference between these lines is significant ($P = 0\ 05$) (Fisher s F)

The detailed analysis of the shape of the curves shown in Figs 3–7 gave interesting results It was found that the experimental curves were in significantly better accordance with a combined cumulative distribution curve consisting of the sum of three than with *one or two* normal distributions (Figs 9 and 10) This point will be further dealt with in the discussion

To compare most correctly the latency times *in the* experimental groups the mean values for the three statistical subpopulations and for the three first papillomas for the *different* concentrations and for the two time intervals are shown in Table 3 and Fig 11 It is seen from Table 3 that the average time of *occurrence of the 3 first* papillomas in

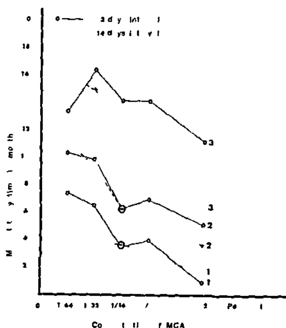


Fig 11

Mean latency time in months for the 3 statistical subpopulation of tumours after 5 repeated paintings with MCA in benzene at different concentrations and applied at intervals of 3 and 14 days respectively The numbers refer to the first the second and the third statistical subpopulations respectively

each group is generally shorter after 14 day intervals than after 3 day intervals Fig 11 and Table 3 show that there is with some few exceptions a similar but moderate difference in the latency time parameters of the first and the second statistical population As regards the third statistical population this difference is considerable for the higher concentrations but with 1/64 per cent as an exception from the trend

Carcinomas

Both in the old and the new series we tried to observe the time of appearance of the carcinomas by noting when there was a palpable subcutaneous infiltration Table 4 and Figs 12 and 13 demonstrate the appearance of the carcinomas in the two groups It is seen that the most striking difference between the old and the new series is the pronounced increase in number of carcinomas per mouse and in the percentage of mice bearing carcinomas when 14 instead of 3-day intervals are used

In Table 4 the mean latency times for the carcinomas occurring after painting with 1/16 1/8 and 1/2 per cent MCA at three day intervals are compared with the latency times of an equal first number of carcinomas occurring after painting at 14 day intervals Thus the use of

TABLE 4

Carcinomas Appearing on Hairless Mouse Skin up to 19 Months after 5 Consecutive Applications of Different Doses of MCA in Benzene Solution Applied at 3 and 14 Day Intervals Respectively

3 Methyl cholanthrene concentration in per cent	Total number of carcinomas per number of mice alive at appearance of first papilloma		Average time of occurrence of the carcinomas (in months)	
	3 day intervals	14 day intervals	3 day intervals	14 day intervals
1/64	0/21	1/2?		
1/?	0/21	6/30		
1/16	1/30	9/30	14 9 (1 ca)	13 0 (The first ca)
1/8	?/2	31/30	16 7 (? ca)	11 6 (The earliest ca
1/?	5/17	22/?8	15 4 (5 ca)	7 3 (The 5 earliest ca

longer intervals even reduces significantly the latency time in all three groups

The Time Period between the Occurrence of First Papilloma and First Carcinoma in Each Animal

In the old and the new series we observed altogether ?? animals in which a papilloma appeared some time before a carcinoma The average time between the first painting and the first papilloma in each single animal was 212 ± 103 days The average time between the first painting and the appearance of the first carcinoma in each animal was 444

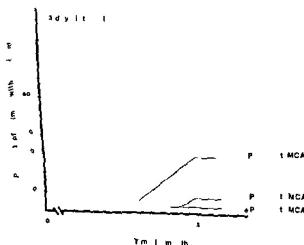


Fig 12

Percentage of carcinoma bearing animals during the observation period after 5 repeated paintings with MCA in *benzene at different* concentrations and with 3 day intervals between the paintings

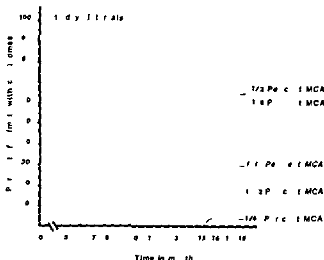


Fig. 13

Percentage of carcinoma bearing animals during the observation period after 5 repeated paintings with MCA in benzene at different concentrations and with 14 day intervals between the paintings

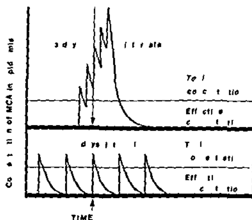


Fig. 14

Schematic and purely speculative drawing of the amount of MCA persisting in the epidermis after the two modes of application. It is assumed that after each painting with MCA the concentration falls and is reduced to a very small amount after two weeks. It is assumed that only a limited amount of carcinogen can act as an effective concentration and that any excess over this limit is either without effect or acts as a toxic concentration. It is seen how much more an effective dose will result from the use of 14 instead of 3 days between the applications

± 84 days. The average time from the occurrence of the first papilloma to the occurrence of the first carcinoma in each single animal was thus 232 ± 100 days. The correlation between the time interval between painting and occurrence of the first papilloma and the time interval between painting and occurrence of the first carcinoma was $R = 0.466$

This means that statistically there is significant correlation between the time of appearance of papilloma and the time of appearance of carcinoma in each single animal ($P = 0.05$)

DISCUSSION

The Difference in Effect between 3 and 14 Day Intervals and the Dose/Response Relationship

This investigation has shown that the *tumourigenic* (papillomas and carcinomas) effect of 5 applications of different doses of MCA to mouse skin is somewhat more pronounced when the applications are given at intervals of 14 days than when intervals of three days are used. The number of tumours per mouse and the percentage of tumour bearing animals increase and the latency time decreases. The observed differences are moderate but probably real. As regards the *carcinogenic* (carcinomas only) effect of 5 applications of MCA applied at different time intervals the investigation demonstrates without doubt that the use of 14 day intervals instead of 3 day intervals increases the number of carcinomas and reduces the latency time both strikingly and significantly. The length of the time interval between the applications is thus of major importance for the development of carcinomas and of some importance for the papilloma development.

The carcinogenic force of a certain amount of a carcinogen is thus more pronounced when the carcinogen is delivered over a longer time period. Is is probably far too early to speculate about possible practical implications of these findings in relation to environmental carcinogenesis in man. But it may be worthwhile to remember that exposure to many smaller doses of a carcinogen over a longer time period might be more dangerous than exposure to higher doses over shorter time intervals.

There are many possible explanations of the observed difference in effect of the two modes of application.

a. The effective dose of carcinogen (*i.e.* the number of carcinogenic molecules that bind to the supposed critical sites in the cells) may have been significantly different. It is very difficult to estimate the true effective dose after a surface application of carcinogen. Certainly in the case of the higher doses an unknown amount of carcinogen is left on the surface and does not penetrate the cells. It may be assumed that the main part of this surface carcinogen is eliminated from the animal by the acute cell loss as demonstrated by *Iversen & Evensen* (1962) and by *Skjæggestad* (1964). How long a carcinogen persists in the epidermis has been estimated differently. After an application a certain amount of it penetrates into the epidermis and some of it further into the corium where a part is resorbed into the general circulation and finally excreted. But some

carcinogen remains in the epidermis for some time after the application This carcinogen is eliminated partly through metabolism in the epidermal cells themselves partly through continuous diffusion into the corium and partly through the loss of carcinogen loaded cells from the surface Different estimates of the time that carcinogens are present in the epidermis after a single application range from one day up to three weeks Most probably there is also a certain correlation between the dose applied and the time of persistence in the skin (*Norden* 1953)

The carcinogen present in the cells may be bound to DNA to RNA to lysosomes to mitochondria or to more unorganized proteins and lipids When three day intervals between the five applications are used the total time of contact between the epidermis and the carcinogen is 12 days + 1 day to 3 weeks With 14 day intervals the total time of contact is 56 days + 1 day to 3 weeks probably involving great fluctuations in the amount present during the painting period (see Fig 14) This longer persistence of carcinogen in the skin may explain the observed differences

It may also be that the cells in the treated mouse skin can respond only to a limited amount of carcinogen (see Fig 14) and that any excess above this limit is unable to influence the carcinogenic process (*Hieger* 1965) Using 3 day intervals there may at the time of each application be so much carcinogen left in the epidermis from the previous application that the new application gives a superfluous of carcinogenic molecules which are of no use This would be most pronounced with the higher concentrations of MCA just as demonstrated in this study Using 14 day intervals the amount applied previously may be reduced to such a degree that a new application can excert a better effect It has also been suggested that skin treated with carcinogens develops a refractory state lasting for some time (*Shubik & Ritchie* 1953) If such a refractory state lasts for more than three days but less than 14 days such a mechanism may explain our observations After new experiments however *Ritchie & Shinozuka* (1967) doubted the existence of such a refractory state in the carcinogen treated skin (see even section d in this text)

It has been shown (*Stjernsward* 1966) that the application of MCA to the skin of mice disturbs the immunological capacity of the animal This inhibition of the immune defense mechanism seems to be of some importance for the tumour yield The depression of immunological capacity may last up to 16 weeks Calculated according to the values given by Stjernsward the reduced immunological capacity in our animals would last for about 18 weeks when 3 day intervals were used and for about 25 weeks when 14 day intervals were

used. This may explain why more tumours develop if the applications are given at 14 day intervals. It may be that the immunological reactivity is of greater importance for the development of carcinomas than of papillomas. Our findings are consistent with this theory.

d. Previous contact with a carcinogen may provoke increasing resistance to the same carcinogen in the epidermal cells (*Vasiliev & Guelstein* 1967). If such an increase in resistance is more pronounced if the intervals between the applications are shorter, the findings may be explained in this way.

e. A general or local direct toxic effect which in some way inhibits the capacity for tumour formation can be imagined to be more pronounced both when the concentration is increased and when the applications are given at 3- rather than at 14-day intervals. Figs. 1 and 2 give some support for this explanation. The relationship between concentration and per cent of tumour bearing animals increases following a straight line for the lowest doses, whereas the augmenting effect of the higher doses is less. In the case of 3 day intervals, the effect even decreases significantly again. This may mean that there are two effects of the carcinogen, as proposed by *Gay* (1965) and by *Hulse* (1967) concerning irradiation carcinogenesis, namely i) tumour initiating effect, and ii) a toxic effect reducing the proliferative capacity of the tissue. Our findings may be explained in this way (see Fig. 14).

f. The epidermis is not in the same condition 3 and 14 days after a carcinogen application. Each application provokes cellular death with inflammation and regenerative reactions (*Iversen* 1964). Three days after an application the epidermis is hyperplastic (*Elgjo* 1966). Is is probable that the increased thickness of the epidermis reduces the effective dose in the basal layer. 14 days after an application, the epidermis has an almost normal thickness again.

g. It has been suggested by *Mottram* (1945) that the effect of a carcinogen application is dependent upon the number of cells in mitosis at the time of application. *Frei & Ritchie* (1964), *Shinozuka & Ritchie* (1967) and *Gelboin* (1967) suggested that the effect of a carcinogen application is dependent upon the number of DNA synthesizing cells in the epidermis during the application. We have at present no information about the number of cells in mitosis or the number of DNA synthesizing cells in the epidermis at the time of each application under the experimental conditions used in this study. No conclusions can therefore be drawn concerning these theories.

It is impossible to give preference to any of the above mentioned explanations Possibly they are all of some importance for the result

Why Use the Time of the Third Application as the Zero Time for Comparison

When three day intervals between each of the five applications are used the total time of treatment will be 12 days When 14 day intervals are used this time will be 56 days It is difficult to know exactly the most correct zero time to be used for a comparison of latency times in these two cases There are three possibilities *ie*

i The start of the treatment Such an assumption is well suited when single applications of different doses are compared or when continuous treatment or initiation followed by croton oil promotion is used The method is obviously unsatisfactory when multiple applications at intervals of different length in the two groups are compared If we had chosen the start of the treatment our first group would have finished the treatment about one and a half month before the second This would have introduced an error in the calculation of the latency time

ii The last day of treatment when both groups had received the total dose If we had used that type of registration the differences we have observed would have been more evident But this would have tended to favour too strongly the results from the 14 day interval experiments

iii The time of the third (the median) application We used this method because we felt that it would give the correct weight both to the differences in the doses given and to the time factor

Are the Appearing Tumours Belonging to One or Several Statistical Populations?

When the skin of mice is painted with carcinogens (once repeatedly or with a strong carcinogen followed by multiple paintings with croton oil) the number of tumour bearing mice always increases with time following a characteristic trend When the results are plotted as percentage of tumour bearing animals versus time (see Figs 3–7) each curve has a shape which resembles a cumulative distribution function

In order to study the mechanism lying behind these curves we have formulated some hypotheses *ie*

i Only a certain percentage of the mice develop tumours This percentage is in some way positively dependent upon the concentration of the applied carcinogen (Figs 1 and 2) as long as moderate doses are used

ii For the tumour bearing animals there will be a certain latency time before the first tumour becomes observable This latency time is also in some way dependent upon the concentration of the applied carcinogen (Fig 11)

iii Because of individual differences in sensitivity of the animals all the tumours will not occur simultaneously In this way the latency times for the first tumours will be distributed according to some frequency function (Figs 3–7)

On these assumptions each of the experimental curves can be described as the sum of one or more statistical populations each with three parameters *ie* the mean value the amplitude and the standard deviation We use a computer program developed at the Sentralinstitutt for Industriell Forskning to find the values of these parameters by the method of least squares We then had to make only one further assumption namely (iv) to choose a suitable frequency function for the individual differences between the animals We could in our case see no objection to the use of a normal distribution

From the parameter values we can draw a curve through the experimental points An example of this concerning the use of 1/16 per cent MCA in benzene is shown in Figs 9 and 10 From these figures two conclusions can be drawn

i A single normal distribution fitted to the experimental data gives a positive probability for time zero (Fig 9) This would have meant that a certain percentage of animals should have tumours at the beginning of the painting This is of course not the fact

ii The calculated curve is not in very good accordance with the experimental points

The positive probability for the time zero can be eliminated by using another distribution which is slightly skewed. The bad fit to the experimental points at the other part of the curve is a more interesting problem. It could be due to experimental errors, but the deviation between the theoretical and the observed curves follows a similar trend in all our experiments. A reasonable improvement can be obtained by extending the original assumptions as follows:

Either

i. The mouse population consists of 2, 3 or more subpopulations, each with its own mean latency time. This is not very likely because this strain of mice is, although not purely inbred, rather homogenetic.

Or

ii. The tumours are of different types, each with its own mean latency time. Such possible interpretations have been commented upon by *Berenblum* (1954) and *Klinken Rasmussen* (1956).

Or

iii. Waves of biological growth processes in the animals may result in waves of tumour appearance. These waves may be hair growth cycles or other fluctuations following hormonal cycles or age cycles in the animals, or it might theoretically even be seasonal variations. The last point has been discussed by *Kreyberg & Schrader Nielsen* (1936).

The mathematical treatment of the observed data is identical for these three hypotheses. Fig. 10 shows an example where three normal distributions are fitted to the same set of data as in Fig. 9. It is clear from these figures that the discrepancy between the theoretical and the experimental results are eliminated in this way. For most of our single experimental curves, and for the whole set of data treated as an unit, we have found a very good agreement between theory and facts in assuming that the curves are the sum of three normal distributions.

Our experiments thus give some support to the theory that tumour development with respect to time after repeated paintings with a carcinogen results in a cumulative frequency distribution curve which seems to be the sum of not *only one* but probably of three statistical subpopulations. We have at present no detailed explanation of this fact. Our data are not sufficiently comprehensive to be *used as a* basis for any conclusion concerning the carcinogenic mechanism. We have been able to see the protocol from the earlier works of *Kreyberg* (1933) and to examine his curves with a digital computer in the same way as described here. Even his curves fit better into a model of three than of one subpopulation. Kreyberg used a strain of purely inbred animals, but he painted his mice continuously with a less well defined carcinogen, namely a crude shale oil product.

The Correlation between the Latency Times of the First Papilloma and the First Carcinoma in the same Animal

It is a common experience in skin carcinogenesis studies that some papillomas regress, some develop into carcinomas and some carcinomas occur without passing through the papilloma stage.

In our material we saw 3 animals with carcinomas without a previous papilloma, 55 had first a papilloma *and* then a carcinoma, and there was a significant correlation between the latency times of these two types of tumour. The other animals had either papillomas exclusively or absence of visible proliferations in *their skin*. It is generally held that the development of papillomas can be used as an index of carcinogenesis. It is interesting to note that *the first* tumours reported by *Yamagiwa & Ichikawa* (1914) obviously were papillomas and kerato achantomas and not cancers. Still their observation was the starting point of modern skin carcinogenesis research.

We feel that the correlation between papillomas and carcinomas in

our material gives some support to the theory that it is valid to use the development of papillomas as an index of carcinogenesis. On the other hand for quantitative purposes papilloma development can be regarded as no more than a very rough index of carcinogenic force.

SUMMARY

Six groups of 30 hairless mice (15 males and 15 females) were given five applications of different exact doses of methylcholanthrene in benzene solution at intervals of 14 days between each application. The tumour development was observed and the results compared to the results of a previous study in which exactly the same doses of methylcholanthrene were given at intervals of three days. The following observations were made, viz.

There was a striking and very significant difference between the two modes of application as regards the yield of carcinomas. The final percentage of carcinoma bearing animals and the mean number of carcinomas per mouse were much higher in the 14 day interval groups and the latency times were much shorter.

The total number of tumours (papillomas and carcinomas considered together) and the mean number of tumours per mouse increased with the dose whether one or the other mode of application were used.

The final percentage of tumour bearing mice varied directly with dose for the lower concentrations but not for the higher concentrations.

The number of tumour bearing animals increased earlier and in some groups to a higher final percentage at 14 day intervals than at three day intervals. These differences were of moderate degree and not entirely consistent.

The final mean number of tumours per mouse was higher and the latency time for the tumour appearance was generally somewhat shorter when 14 day intervals were used than when three day intervals were used.

The cumulative distribution curves of percentage of tumour bearing animals versus time were analysed with an electronic computer. It was found that the curve fit was much better if the observed curve were assumed to be the sum of three normal statistical populations rather than of one or two.

A significant correlation between the time of appearance of the first papilloma and the first carcinoma was found in each single animal in a selected group of 55 animals that had a papilloma before they got a carcinoma.

Different possible explanations of the observed facts are discussed and a possible implication in relation to environmental carcinogenesis in man is mentioned.

The Following Conclusions are Drawn Viz

The length of the time interval between the applications is of major importance for the development of carcinomas and of some importance for the papilloma development

There is a tendency to a straight line correlation between the logarithm of the dose and the tumour yield for the lower concentrations With higher concentrations of methylcholanthrene the tumour yield decreases again

The tumours seem to belong to three statistical subpopulations

The development of papillomas may be used as an index for carcinogenesis but only as a rough estimate and not for quantitative purposes

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PERICARDIAL ABSORPTION OF EXOGENOUS PEROXIDASE IN RATS

An Electron Microscopical Study

By

TROND KLUGE and TORSTEIN HOVIG

Received 17 1 68

Particulate matter such as colloidal thorium dioxide (11) colloidal gold and iron saccharate (15) is absorbed through the cytoplasm of pericardial mesothelial cells None of these tracer substances have been observed inside the intercellular spaces It has been claimed that the tight junctions (zonulae occludentes adhesion belts) seal off the spaces between adjacent cells in various tissues (4 14 16) However intercellular passage of ions and small molecules has been observed in some endothelia and mesothelia (1 3 5) Moreover from the absorption capacity of capillaries it has been deducted that intercellular pores or openings must be present (13)

The mechanisms of protein absorption from the pericardial cavity are poorly understood (19 20) and electron microscopic studies on the subject have not been reported However the recent introduction by *Karnowsky et al* (6 7) of a new histochemical method has added greatly to the knowledge of protein absorption on the ultrastructural level This method employs the use of exogenous peroxidase which gives an electron dense histochemical product when reacting with benzidine and osmium tetroxide (6) A modification of the method has been used in the present study with the following purpose

1 To observe the absorption of protein by the parietal and visceral pericardial mesothelium
2 To study the mechanisms of absorption with particular reference to the intercellular spaces and junctions

MATERIALS AND METHODS

Horseradish peroxidase type II (Sigma Chemical Co St Louis Missouri U S A) This is a haemoprotein with a presumed particle size of about 3 mmy and a molecular weight of about 40 000 (8) It contains four or more fractions which can be

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separated by electrophoresis but the major components do not differ significantly in molecular weight amino acid composition or enzymatic activity (9) Its reaction product with benzidine and osmium tetroxide has a high electron opacity and is easily identified

The protein solution was made by dissolving 10 mg of peroxidase in 2 ml of normal saline A standard volume of 0 3 ml was injected into the pericardial cavity of each laboratory animal

Experimental procedure Twenty four adult male and female rats weighing between 250 and 350 g were anaesthetized with ether/alcohol (2 1) through an endotracheal catheter (12) The chest was opened and the peroxidase solution was injected into the pericardial cavity The lung was re expanded and the thoracotomy closed For intervals longer than 15 minutes between injection and fixation the animals were awake with free access to food and water for shorter intervals they were kept under continuous anaesthesia

Fixation and sampling of tissue was carried out at varying time intervals from 1 minute to 2 hours following injection Thin slices were cut out from the parietal and visceral pericardium (10)

Tissue preparation The specimens were fixed in ice cold 2 5 per cent glutaraldehyde for 2–3 hours They were washed overnight in 0 1 M phosphate buffer containing 5 per cent sucrose and then incubated for 5 minutes in 5 mg of 3–3 diaminobenzidine tetrahydrochloride (Sigma Chemical Co) dissolved in 10 ml of Tris HCl buffer at pH 7 6 The specimens were washed in 3 changes of distilled water and postfixed for 2 hours in osmium tetroxide

In specimens from 3 additional animals glutaraldehyde fixation was omitted This tissue was briefly rinsed in phosphate buffer and incubated in the benzidine solution as described above followed by fixation in osmium tetroxide

All specimens were dehydrated in graded alcohols and embedded in Epon 812 Ultrathin sections were cut on Huxley and LKB microtomes stained with uranyl acetate and lead citrate and examined in a Siemens Elmiskop I

Controls To exclude artefactual adsorption of peroxidase to the mesothelium specimens from four non injected animals were fixed for 2 hours in glutaraldehyde washed in buffer and then soaked in the peroxidase solution for 60 minutes They were washed in distilled water transferred to the benzidine solution postfixed in osmium tetroxide and processed as previously described

RESULTS

The experiment was well tolerated by all animals No signs of toxic reactions were observed Local cell damage to the mesothelium did not occur as judged from the electron micrographs (vide infra)

A Parietal Mesothelium

During the first 10 minutes following injection of peroxidase the mesothelium showed no evidence of protein absorption The intercellular spaces displayed the usual low electron density and the cellular contact areas were clearly outlined (Figs 1 4) At 15 minutes the intercellular spaces were found to contain reaction product (Figs 7 and 8) which possessed a high electron density and frequently showed a mottled appearance The concentration of reaction product seemed to increase during the next 45 minutes when the intercellular spaces appeared densely filled with disorbed material (Figs 9 13) Commonly the reaction product occupied the entire length of the intercellular space including the areas of the maculae adherentes (desmosomes) (Figs 9 and 11) At 90 minutes some intercellular spaces were devoid of reaction product and at 2 hours they all appeared empty (Fig 20)

Most of the tight junctions displayed a normal diameter (Figs 8 10 and 12) with apparently fused membranes of opposing cells In some few junctions an increased distance between adjacent plasma membranes was observed (Figs 5 and 6) This apparent gap between opposing cells was occasionally seen during the initial stages of absorption and seemed present following both types of fixation

At 30 and 45 minutes reaction product was present inside a few of the vesicles and vacuoles in the cytoplasm The appearance of these vacuoles was variable from those containing sparse irregular deposits about the periphery (Fig 14) to those filled with dense homogenous reaction product (Fig 15) A few of these vacuoles were in communication with the pericardial lumen or opening into the subcellular space Most of the stained vacuoles however were in communication with the intercellular spaces (Figs 11 12 26 and 27) The majority of cytoplasmic vesicles and vacuoles appeared empty at all stages of absorption (Figs 9 11 14 and 15)

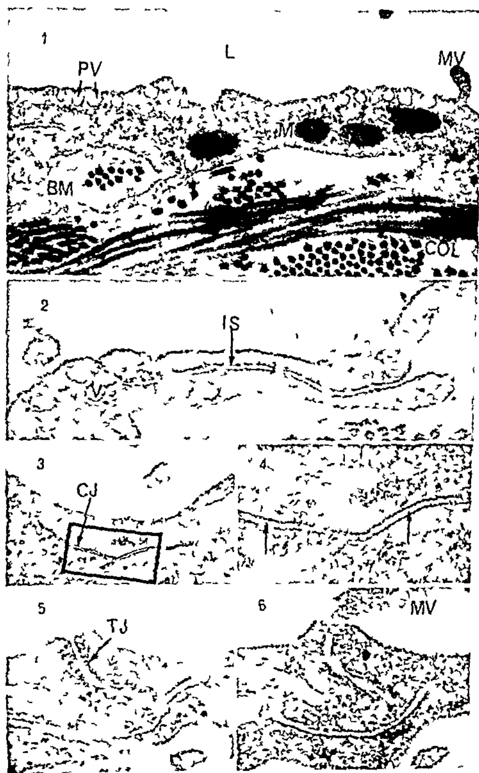
From 45 minutes onwards reaction product was observed also inside some of the electron dense bodies in the cytoplasm (Figs 16–20) Due to the high electron density and mottled appearance of the reaction product it was sometimes difficult to distinguish between a dense body and several vacuoles filled with reaction product (Figs 18 and 19) On several occasions a distinct membrane encircling the reaction product could not be outlined Such protein droplets were frequently lying in close proximity in the cytoplasm or even appeared to merge with each other (Figs 16 and 19) Sometimes the possibility of intermediate stages between vacuoles and dense bodies was considered (Figs 16 18 and 19)

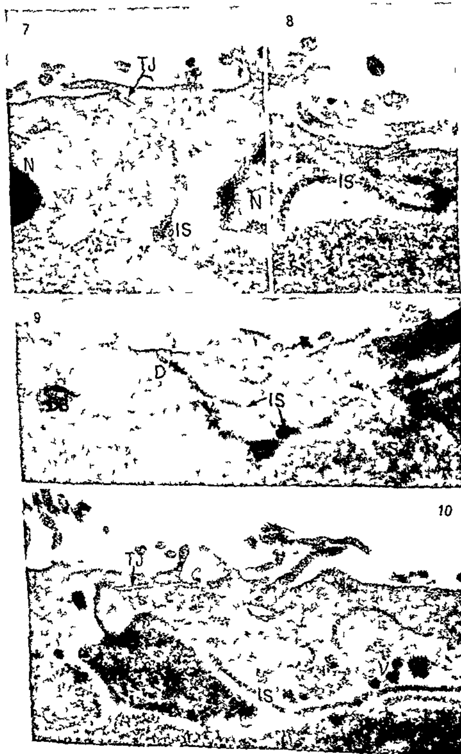
The basement membrane appeared in most sections as a continuous structure Reaction product filling the intercellular spaces frequently extended underneath the adjacent cells thus occupying the subcellular space When absorption seemed to be at its maximum the whole submesothelial area was filled with reaction product thus making the basement membrane indistinguishable (Figs 9–11)

B Visceral Mesothelium

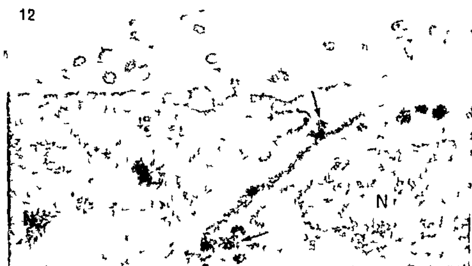
In the visceral mesothelium reaction product was not observed until 30–45 minutes following injection of peroxidase Up to this period the intercellular spaces appeared normal with a low electron density (Figs 21 and 22) When reaction product appeared its localization and progressive concentration seemed identical with its behaviour in the parietal mesothelium (Figs 23–28) The amount of protein present however did not appear as heavy as in the parietal membrane

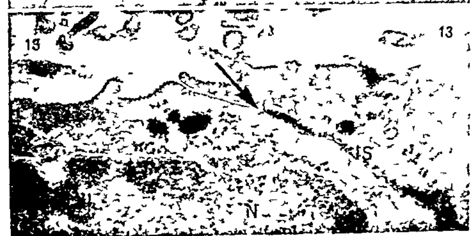
The visceral cells containing an abundant endoplasmic reticulum usually showed a normal appearance (Figs 21 24 and 28) Occasionally the reticulum showed irregular dilatations (Fig 22)


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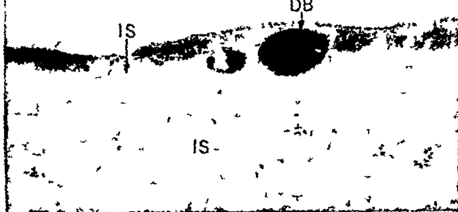

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Figs 1–20

Parietal mesothelium at varying time intervals following injection of peroxidase into the pericardial cavity

Fig 1 5 minutes after injection Normal appearance of mesothelial cell with micro villi (MV) pinocytic vesicles (PV) and mitochondria (M) The basement membrane (BM) separates the mesothelium from the subcellular area con taining collagen (COL) Pericardial lumen (L) × 30 000

Fig 2 10 minutes The intercellular space (IS) between two mesothelial cells appears empty Some cytoplasmic vesicles (V) communicate with the inter cellular space × 45 000

Figs 3 and 4 10 minutes This cell junction (CJ) displays a long closed segment where the opposing plasma membranes seem to fuse into a central line (arrows) Fig 3 × 45 000 Fig 4 × 75 000

Figs 5 and 6 15 minutes Occasionally the tight junctions (TJ) present an increased diameter giving the impression of a gap between adjacent cells Microvillus (MV) Serial sections × 75 000

Figs 7 and 8 15 minutes Darkly stained reaction product is present along the inter cellular spaces (IS) beneath the tight junctions (TJ) and in the submeso thelial area Nuclei (N) × 45 000

Fig 9 30 minutes Increasing amounts of reaction product inside the intercellular space and in the submesothelial area (SA) The cytoplasmic structures still seem devoid of tracer substance Desmosome (D) Dense body (DB) × 30 000

Fig 10 45 minutes A tortuous intercellular space (IS) displays a wide segment con taining heavy amounts of reaction product Some cytoplasmic vacuoles (V) also show specific staining The vacuoles may communicate with the inter cellular space at another plane of section (see text) Tight junction (TJ) × 45 000

Fig 11 45 Minutes Maximum of absorption into the intercellular space (IS) Most cytoplasmic vesicles appear empty but those communicating with the inter cellular space (arrows) are filled with reaction product Nuclei (N) × 21 000

Figs 12 and 13 60 minutes Still considerable amounts of reaction product in the intercellular spaces (IS) from the lower margin of the tight junctions (heavy arrow) Several vacuoles (small arrows) communicate with the intercellular space Nuclei (N) Fig 12 × 30 000 Fig 13 × 45 000

Figs 14 and 15 Cytoplasmic absorption of peroxidase 45 minutes Some vesicles (arrows) contain sparse amounts of reaction product (Fig 14) while others are densely stained (Fig 15) Most vesicles and vacuoles (V) are devoid of tracer substance × 45 000

Fig 16 60 minutes Several protein droplets (arrows) in the cytoplasm (see text) A dense body (DB) probably contains small amounts of reaction product × 45 000

Fig 17 60 minutes High magnification of dense body containing scattered deeply stained material A nearby vacuole (V) appears to be filled with reaction product × 75 000

Fig 18 90 minutes Dense body containing numerous droplets of reaction product giving the appearance of a multivesicular body × 75 000

Fig 19 90 minutes Accumulations of reaction product (arrows) in the cytoplasm These may represent aggregates of vacuoles or multivesicular dense bodies (see text) Mitochondria (M) × 18 000

Fig 20 2 hours Reaction product is retained inside dense bodies (DB) while the intercellular space (IS) is free from tracer substance × 45 000

Figs 21–28

Visceral mesothelium at different time intervals

Figs 21 and 22 15 minutes The cytoplasm and intercellular spaces do not show evidence of peroxidase absorption The rich endoplasmic reticulum (ER) is occasionally dilated (Fig 22) Several empty vacuoles communicate with the intercellular space (arrows) Tight junction (TJ) Desmosomes (D) Fig 21 × 27 000 Fig 22 × 18 000

Fig 23 30 minutes The intercellular space (IS) probably contains traces of reaction product above and below a desmosome (D) × 30 000

Fig 24 45 minutes Definite presence of reaction product along the intercellular space (IS) Endoplasmic reticulum (ER) Scattered ribosomes (R) Dense body (DB) × 30 000

Fig 25 60 minutes Reaction product inside the intercellular space (IS) and in some adjacent vesicles and vacuoles × 30 000

Figs 26 and 27 60 minutes Serial sections demonstrate that most of the labelled vacuoles are in communication with the intercellular space A dense body (arrow) also contains reaction product × 30 000

Fig 28 90 minutes Reaction product is still present along the intercellular space (IS) and in vacuoles and dense bodies (DB) in the cytoplasm Nuclei (N) Endoplasmic reticulum (ER) × 21 000

C *The Submesothelial Tissue*

Occasional mast cells were observed immediately underneath the mesothelium of both surfaces They were well preserved and displayed granules of normal appearance and without signs of disruption On two occasions a mast cell was lying immediately underneath a cell junction which appeared normal apart from the presence of reaction product in the intercellular space The diameter of the tight junction on top of the intercellular space was not increased as judged from serial sections

Some of the macrophages in the submesothelial tissue were seen to contain reaction product usually inside electron dense bodies Fibroblasts and leucocytes did not appear to participate in peroxidase absorption

DISCUSSION

The employment of frozen sections for incubation in benzidine as used by *Karnovsky et al* (6) was omitted in the present study It was expected that the thin pericardial membranes would be sufficiently exposed to the protein solution by surface application alone In addition an accurate localization of surface cells is far more easy in unsectioned specimens than in tissue already sectioned in one direction It was also considered an advantage to avoid artefacts possibly induced by freezing

There was no evidence of artefactual adsorption of peroxidase as the control specimens showed no specific staining This agrees with the results obtained by *Graham & Karnovsky* (6) and indicates that the observations reflect an *in vivo* absorption of protein

The first evidence of absorption was observed in the parietal mesothelium 15 minutes after injection of peroxidase Considerable increase in absorption followed *during the next 15–30 minutes In the visceral* mesothelium absorption was delayed and appeared to reach its maximum in 60–90 minutes This lag period between parietal and visceral absorption has also been noted with respect to particulate matter (11) and supports the theory that the main fluid stream in the pericardial cavity is directed towards the parietal membrane

Of particular interest is the observation of reaction product inside

the intercellular spaces Four different possibilities exist for absorption along this pathway

1 The application of peroxidase may exert an influence upon the tight junctions and separate their adjacent membranes
2 Peroxidase may by pass the tight junctions by means of cytoplasmic vesicles and vacuoles
3 The tight junction may not encircle each cell as a complete adhesion belt
4 The tight junctions may be permeable to the peroxidase molecules

It is known that peroxidase may act as a histamine liberator when given intravenously to mice (18) In the present study however no signs of toxic reactions in the animals were observed It was also considered unlikely that generalized phenomena should be induced by the application of very small amounts of peroxidase to a local surface

Concerning a direct influence of peroxidase upon the diameter of the tight junctions *Karnovsky* (7) argues against this possibility His view is supported by the present study as the tight junctions with very few exceptions displayed a normal diameter It should be noted that the mast cells in the submesothelial area presented normal granules with intact membranes and that nearby cell junctions also showed a normal morphology These observations speak against a local liberation and influence of histamine upon the tight junctions

As the intercellular spaces are known to communicate with some of the cytoplasmic vesicles and vacuoles (10) the spaces might possibly receive some peroxidase through this pathway In the present study however intercellular absorption occurred earlier than absorption into the cytoplasmic vacuoles It also appeared from the micrographs that most of the stained vacuoles were filled from the intercellular spaces In addition the amount of reaction product present in cytoplasmic vacuoles seemed far too small to explain the heavy protein absorption into the intercellular spaces and the subcellular area

Tight junctions are reported to be absent between some vertically sectioned cells in mesothelium (10) and in small lymphatics (2) Hence if they surround the cells as incomplete circles particles may pass through the interruptions and into the intercellular spaces This possibility cannot be excluded on the basis of findings in the present study It should be stressed however that the absence of tight junctions in normal pericardial mesothelium is an infrequent observation (10)

In the present experiments the intercellular spaces were the first structures to be labelled with peroxidase reaction product As a rule the spaces were filled with protein along their entire lengths from the inferior margin of the tight junctions Accordingly the most likely explanation of the present observations is that the tight junctions in fact

are penetrable to peroxidase. This view is in accordance with the recent observations on endothelial absorption of protein reported by *Karnovsky* (7). He also favours the theory of passage through the junctions of adjacent endothelial cells and suggests that the tight junctions are not completely tight and should be termed maculae rather than zonulae occludentes.

It is a commonly accepted theory (3) that substances with a molecular weight above 20 000 cannot pass through intercellular spaces in mesothelium. However, the present observations clearly indicate that protein molecules with a MW of 40 000 can be absorbed along this pathway.

Reaction product was present also inside some vacuoles without apparent communication with the intercellular spaces. Since these organelles were opening at both plasma membranes (10), it is possible that a minor portion of protein crossed the cells inside vacuoles. Transcellular transport along this pathway has previously been established for particulate matter (1, 5, 11) and is considered for protein absorption through endothelium (7).

In general, however, the main absorption of peroxidase seemed to take place through the intercellular spaces, while colloidal thorium dioxide (11) was absorbed exclusively through the cytoplasm of the mesothelial cells. The size of the tracer particles may be of importance to this principal difference in absorption mechanism. While peroxidase is believed to have a particle diameter of about 30 Å, the corresponding figure for thorium dioxide is 100–300 Å. It has been suggested that the average width of a "tight" junction is about 40–60 Å (7), thus allowing the passage of particles with a smaller diameter.

The many biological and chemical differences between protein and thorium particles also may account for differences in absorption. It seems likely, for instance, that thorium dioxide is a potent stimulant for mesothelial phagocytosis, while this mechanism may be of less importance to the absorption of protein.

The incorporation of peroxidase reaction product into some of the electron dense cytoplasmic bodies indicates that these structures also participate in the absorption of protein. It has previously been suggested (11) that the dense bodies represent lysosomes, phagosomes (phagocytic vacuoles) or phagolysosomes. The observations of *Straus* (17, 18) also indicate that a high affinity exists between lysosomes and exogenous peroxidase. Furthermore, the present observations support the theory of an interaction between cytoplasmic vacuoles and the electron dense bodies (11).

The rapid occurrence and heavy accumulation of reaction product in the submesothelial area suggests that the basement membrane does not act as a barrier to pericardial absorption of peroxidase molecules.

SUMMARY

Absorption of protein through pericardial mesothelium was studied on the ultrastructural level by injecting a solution of peroxidase into the pericardial cavity of rats Following initial fixation the absorption sites were labelled by treatment with benzidine and osmium tetroxide

Absorption started earlier and seemed more heavy in the parietal than in the visceral membrane indicating that the main fluid stream in the pericardial cavity is directed towards the parietal membrane

Peroxidase was absorbed mainly between the mesothelial cells and filled the intercellular spaces along their entire lengths As opposed to previous theories the present observations indicate that substances with a molecular weight of 40 000 and a particle size about 30 Å may pass through the intercellular junctions It is suggested that the tight junctions are penetrable to protein molecules of this order

Smaller amounts of reaction product were observed inside some of the cytoplasmic vacuoles and inside electron dense bodies interpreted as lysosomes or phago lysosomes

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CELL INFILTRATIONS IN THE PANCREAS OF NEWBORN INFANTS OF DIABETIC MOTHERS

By

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Infiltrates of lymphocytes and haemopoietic cells often occur in the pancreas of foetuses and newborn infants (*Potter* 1961 *Liu & Potter* 1962) In the offspring of diabetic mothers the pancreas appears to be unique in two respects A marked hypertrophy of the islets of Langerhans is regularly observed and in addition conspicuous infiltrates consisting of eosinophilic cells in the connective tissue surrounding the islets have been noticed by several investigators (*Helwig* 1940 *Cardell* 1953a *Driscoll Benirschke & Curtis* 1960 *Silverman* 1963 *D Agostino & Bahn* 1963) Charcot Leyden crystals within these eosinophilic infiltrates have been described (*McKay Benirschke & Curtis* 1953 *Silverman* 1963 *D Agostino & Bahn* 1963 *Warren & Le Compte* 1966)

Hypertrophy of the pancreatic islet tissue as well as infiltrates of haemopoietic cells in newborn infants with erythroblastosis foetalis have also been described (*Miller Johnson & Durlacher* 1944 *Potter Seckel & Stryker* 1941 *Brahler & Dallenbach Hallweg* 1963)

The aim of the present study is to investigate the frequency and the extent of eosinophilic cells haemopoietic cells and accumulations of lymphocytes in the pancreas of newborn infants of diabetic mothers and in infants with erythroblastosis foetalis and to compare the findings with similar results in a control material The incidence and degree of cell infiltrations as well as the degree of islet hypertrophy were related to birthweight gestational age hours of life and degree of maternal diabetes or immunization in the infant

MATERIAL AND METHODS

The material consisted of pancreatic glands from 48 infants of diabetic mothers from [illegible]0 infants with rhesus immunization and from 6[illegible] in a control group in which the mothers according to the clinical record had neither diabetes nor rhesus immunization With the exception of 5 cases from the period 1947–[illegible] the diabetic

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group was taken from a consecutive autopsy series from the period 1960 to 1967 excluding only macerated infants and cases in which the pancreas showed marked post mortem autolysis. All infants were born in the obstetrical departments Rigshospitalet Copenhagen. The erythroblastosis and the control material were selected from the same autopsy series from the years 1964–66 with special reference to birthweight and length of postnatal life in order to get materials comparable to the diabetic material as regards these parameters.

Most of the pancreases were fixed in 10 per cent buffered neutral formalin but a few were fixed in Bouins solution. In about 25 per cent of each group the total pancreas was fixed and longitudinal sections used. The remaining blocks mostly consisted of tissue from the pancreatic body and tail. All paraffin blocks were recut and stained with haematoxylin eosin, Van Gieson Hansen stain, Pyronin methyl green, methylviolet and periodic acid Schiff (PAS) technique. Sections from the infants of diabetic mothers and a few from the two other groups were stained with acid fuchsine in order to demonstrate Charcot Leyden crystals.

Haematoxylin eosin, Van Gieson Hansen and PAS stained sections were used in the subjective grading of cell infiltrations and islet hypertrophy and 8 non serial sections from each infant were studied. This grading was done 3 times by one of us (L.M.P.) at intervals of 3–4 months; the results showed only small differences. In these cases the mean result was used. Four categories were used to express the degree of eosinophile and haemopoietic cell infiltrations and islet hypertrophy (Grade 0 = —, Grade 1 = +, Grade 2 = ++, Grade 3 = +++). Zero means absence of the cells in question or a few of these might be scattered and grade one stands for a few (at least two) small infiltrations of more than 10 and less than 50 cells of each type. Grade two reflects several cell infiltrations of more than 50 cells, while in grade three the cell infiltrations occur in nearly all parts of the pancreas. Lymphocyte infiltrations were designated as absent or present (— +). To be described as hypertrophied the islets should be increased both in size and number. The grading was subjective and in cases in which one grading differed from another the sections were compared with several control pancreases in the same weight group.

RESULTS

In Table 1 the clinical data and results of our findings in the diabetic group are given. White's (1949) prognostic classification (with regard to the foetus) of pregnant diabetics was used. Group A – Diabetes treated with diet alone. Group B – Insulin treated diabetes diagnosed at the age of 20 or later with no late diabetic complications. Group C – Diabetes diagnosed before 20 years of age, nearly always in the age group 10–19, and with no late diabetic complications. Group D – Diabetes with nonproliferative retinopathy. Group E – Not used. Group F – Vascular nephropathy and/or proliferative retinopathy.

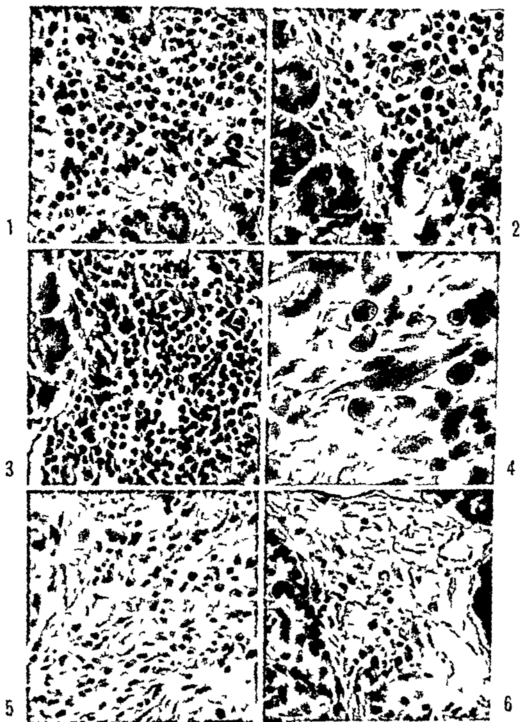
Complications during pregnancy in the form of toxaemia, clinical pyelonephritis, precoma, severe acidosis or haemorrhage occurred in 30 patients or 63 per cent of the diabetic mothers. In all diabetic cases the birthweight ranged from 230 to 5000 g, the gestational age from 23 to 39 weeks, and duration of life from 0 hours to 66 days. Eight infants died *in utero* a few hours before or during delivery. Sixteen infants had major congenital malformations.

In the erythroblastosis group the birthweight ranged from 900 to 4250 g, the gestational age from 28 to 40 weeks. Four infants died *in utero* just before delivery and among 16 liveborn 3 died a few hours after birth from severe hydrops foetalis, while 1–4 exchange trans

TABLE 1

Clinical Data and Degree of Cell Infiltrations and Islet Hypertrophy in the Diabetic Group

Mother Case record no	White class	*Infant Birth weight g*	Gestational age days	*Hours of life*	Islet hypertrophy	Cell infiltrations: *Eosinophile*	Haemopoietic	Lymphocytes
142/66	F	230	160	0	—	—	+	—
334/65	B	750	190	1	+	—	++	—
75/67	C	900	190	30	—	—	+	—
1711/60	C	990 (twins)	220	4	+	+	+	+
1711/60	C	1020	2 0	4	+	+	+	—
2038/61	F	1025	190	72	—	—	++	—
1730/60	C	1100	200	48	—	—	+	—
2177/61	F	1250	206	34	—	—	+	—
1729/63	F	1200	239	0	+	—	++	+
2105/63	C	1300	190	15	+	—	++	—
2120/65	D	1500	220	0	++	++	+	—
568/67	D	1510	225	0	+	—	++	+
1446/47	B	1550	215	5	+	+	—	+
1703/66	D	1600	253	31	+	—	+	—
916/60	I	1650	220	48	—	—	+	—
949/65	D	1650	220	1	—	—	+	+
1309/65	D	1650	225	0 3	++	+	++	+
1749/64	B	1700	210	8	++	+	++	+
207/66	C	1750	205	30	—	—	—	—
984/63	D	1800	247	2	—	—	+++	—
1968/61	F	2000	252	68	—	—	+	—
1979/65	F	2150	248	0	+	—	—	—
2272/61	A	250	237	0	—	—	++	—
1289/66	D	2300	228	17	+	—	+	+
797/65	C	2400	230	10	+++	+++	+	—
1223/60	F	2500	255	10	+++	++	++	—
2324/63	D	2500	220	0 1	+	—	+	—
2401/60	B	2700	232	0	+++	—	+	—
1510/64	F	2700	251	26	+	+	+	—
171 /66	A	2750	268	80	++	—	—	+
1324/47	D	2800	267	132	+	—	—	—
667/50	D	2800	246	5	+	+	+	—
2194/60	B	2850	250	17	+++	+++	++	+
648/64	C	3000	250	17	++	—	+	—
203/61	D	3100	263	0	++	++	+	—
843/61	D	3300	262	13	+	+	+	+
1599/61	D	3400	243	6	++	++	++	+
203/67	D	3400	255	18	+	—	+	+
430/52	D	3400	247	41	++	++	+	+
2203/65	B	34 0	247	13	++	++	+	+
2156/6	A	3500	271	33	+++	+++	—	—
1438/66	D	3900	252	0 6	+++	—	+	+
1182/67	B	3900	260	74	+	—	—	+
1153/65	D	4100	253	216	+++	—	—	—
363/65	B	4150	255	96	+++	—	—	—
561/65	B	4300	250	156	+++	—	+	—
1192/67	F	4450	251	66 days	+++	++	++	+
17 4/47	F	4850	250	11	++	—	++	+
	B	5000	250	0				


1
2
3
4
5
6

fusions were carried out in the remaining 13 One infant had a major congenital malformation

The corresponding control group was selected to include infants of birthweights from 320 to 4700 g and gestational ages from 22 to 43 weeks 20 infants were stillborn and major congenital malformations occurred in 7 infants

As seen in Fig 1 the eosinophilic infiltrates consist of eosinophile polymorphonuclear granulocytes with an intermingling of a few scattered lymphocytes polymorphonuclear neutrophile leucocytes and more immature cells The eosinophile cells are mainly found in the connective tissue surrounding the hypertrophied islets but a few may be seen scattered in all parts of the pancreas The distribution of eosinophile cell infiltrates varies from one part of the pancreas to another Thus hypertrophied islets without eosinophilic infiltrations in the surroundings are nearly always found In infants of non diabetic mothers and infants with erythroblastosis foetalis eosinophile granulocytes are rarely seen and infiltrates consisting of more than 10 cells have not been found

Haemopoietic cell infiltrations (Fig 2) occur with an increasing frequency with decreasing birthweight and gestational age This applies to all groups of infants In Fig 7 the frequency and degree of haemopoietic cell infiltrates in the 3 groups of infants are given As extra medullary haemopoiesis disappears after a few days of life infants with a duration of life longer than 4 days were excluded in all groups In all of the infants with erythroblastosis foetalis this type of cell infiltration occurs to a degree higher than that in the other groups No correlation between haemopoieses and grade of immunization was found when immunization was expressed by bilirubinaemia and haemoglobin at birth and changes in these values in the first 24 hours of life

Compared with the control group haemopoietic cell infiltrations occur more often and to a higher degree in infants of diabetic mothers Haemopoietic cell infiltrates of grade 2 and 3 are more frequent in a diabetic group than in a non diabetic group of comparable gestational

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Figs 1–6

Fig 1 Eosinophile cell infiltration (Haematoxylin eosin × 400)

Fig 2 Infiltration of haemopoietic cells (Haematoxylin eosin × 400)

Fig 3 Lymphocyte infiltration (Haematoxylin eosin × 400)

Fig 4 Charcot Leyden crystals within eosinophile cell infiltration (Acid fuchsine × 1000)

Fig 5 Cell infiltration containing preserved eosinophile cells as well as eosinophile cells in degeneration (Haematoxylin eosin × 400)

Fig 6 Small infiltrate of eosinophile cells in degeneration (Haematoxylin eosin × 400)

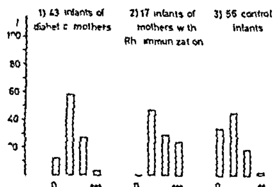


Fig 7
Haemopoiesis in the pancreas in 3 groups of newborn infants

age (Table 2 group I) Looking at this in another way an almost identical distribution of the degree of haemopoietic cell infiltrates may be found in a non diabetic group of shorter mean gestational age (23 days less) and of considerably lower mean birthweight (1200 g lower) (Table 2 group II)

Fig 3 shows a typical lymphocyte infiltration Our control material presented a maximum frequency of lymphocyte infiltration about 6-7 weeks before term while the occurrence was only 20 per cent in 30 infants with a mean gestational age of about 39 weeks In the diabetic group however 11 out of 21 (52 per cent) with a mean gestational age of 37 weeks had lymphocyte infiltrations in the connective tissue

The occurrence of islet hypertrophy is shown in Fig 8 In the control group islet hypertrophy was found in 8 cases but only to a high degree in one infant and the clinical record of the mother of the latter was inadequate as a basis on which to exclude diabetes Ten infants with rhesus immunization had islet hypertrophy of grades 1 and 2 but none of them presented the highest degree In the control and erythro

TABLE 2
Haemopoiesis in the Pancreas of Newborn Infants of Diabetic and Non Diabetic Mothers

	No of infants	Mean gestational age days	Mean birth weight g	Grade of haemopoiesis		
				—	+	++ and +++
Non diabetic group I	30	248	2440	7(23%)	20(67%)	3(10%)
Diabetic group	30	245	2885	6(20%)	15(50%)	9(30%)
Non-diabetic group II	30	222	1680	4(13%)	15(50%)	11(37%)

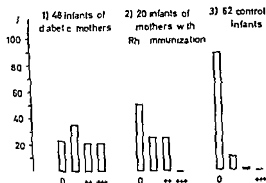


Fig 8
Islet Hypertrophy in 3 groups of newborn infants

blastosis groups the islet hypertrophy could not be related either to birthweight or to any maternal disease

Among infants of diabetic mothers a positive correlation between birthweight and islet hypertrophy was found

In the present material Charcot Leyden crystals were observed in all infants with eosinophile infiltrates grades 2 (7) and 3 (3) and in 2 (25 per cent) with grade 1 (Fig 4) Furthermore in all infiltrates with crystals we noted larger eosinophile cells some of which contained irregular hyperchromatic nuclei decomposed granula and besides they had more or less indistinct cell membranes (Fig 5) These cells were probably eosinophile cells in degeneration

In addition similarly degenerated eosinophile cells were found in 7 infants of diabetic mothers (case record no 1712/66 1324/47 1438/66 1182/67 2153/65 363/65 564/65) with islet hypertrophy but without ordinary eosinophile cell infiltrations (Fig 6) In three of these Charcot Leyden crystals were also present The mean duration of life of the 17 liveborn infants with ordinary eosinophile infiltrations was 10 hours while it was 112 hours in the group in which only degenerated eosinophiles were found

TABLE 3
Eosinophile Cell Infiltrations in Relation to the Grade of Islet Hypertrophy

	Grade of islet hypertrophy			
	0	+	++	+++
Total no of infants	11	17	10	10
Infants with eosinophile or degenerated eosinophile cell infiltrations	0	8(47%)	8(80%)	9(90%)

TABLE 4

Grade of Eosinophile Cell Infiltration in Relation to Birthweight and Gestational Age in 39 Liveborn Infants of Diabetic Mothers

	Grade of eosinophile cell infiltrations			
	0	+	++ and +++	Only degenerated eosinophile cells
No of infants	15	8	10	7
Mean birthweight g	1635	1830	3330	3780
Mean gestational age weeks	31	33	36	37

Eosinophile cell infiltrations Charcot Leyden crystals and degenerated eosinophile cells occurred only in infants of diabetic mothers with islet hypertrophy Table 3 demonstrates a positive correlation between the grade of islet hypertrophy and eosinophilia Three infants with islets hypertrophy of grades 2 and 3 and absence of eosinophile cells were all stillborn therefore only liveborn infants are included in Table 4 According to this table it is likely that a positive correlation also exists between eosinophile cell infiltrations and birthweight as well as gestational age No infant with a gestational age less 32 weeks had eosinophile cell infiltrations

Any correlation between eosinophile infiltrations and the severity of maternal diabetes or the parity of the mother could not be found

DISCUSSION

The positive correlation between the degree of islet hypertrophy and the occurrence of eosinophile cell infiltrations in the pancreas of newborn infants of diabetic mothers (Table 3) speaks in favour of a direct connexion between these abnormalities A positive relation between islet hypertrophy and birthweight exists and a positive relation between birthweight and eosinophile cell infiltrations is demonstrated (Table 4)

Infants with eosinophile infiltrations had a mean duration of life of 10 hours but in a group of infants with a longer mean duration of life (5 days) small infiltrates of degenerated eosinophile cells occurred These infants had islet hypertrophy but no ordinary eosinophile infiltrations It is possible that ordinary eosinophile infiltrations might have been present at the time of birth This could be in keeping with the negative correlation between hours of postnatal life and the degree of eosinophile infiltrations found by *Silverman* (1963)

Infants of diabetic mothers as a group have higher plasma insulin at birth and a much greater reactivity of insulin release in response to intravenous glucose (*Baird & Farquhar* 1962 *Stimmler, Brazie & O Brien* 1964 *Jorgensen Deckert Molsted Pedersen & Pedersen* 1966)

Also the disappearance rates of glucose (k values) after an intravenous glucose load is higher than in normal newborn infants (*Baird & Farquhar* 1962 *Euler Larsson & Persson* 1964 *Molsted Pedersen & Pedersen* 1967) Furthermore *Steinke & Driscoll* (1965) found a higher extractable insulin content in the pancreas from infants of diabetic mothers

In the hypertrophied islet tissue of infants of diabetic mothers the beta cells contain enlarged polymorphic and hyperchromatic nuclei morphological signs which probably express hyperactivity of the cell According to *Hultquist & Olding* (1967) the nuclear size of the beta cells was significantly increased immediatly after delivery while there was no nuclear enlargement in infants more than 4 days old This is in keeping with the neonatal changes in the disappearance rate of glucose (k value) (*Molsted Pedersen* 1968) according to which the k value is high at birth but at the same level as in infants of non diabetic mothers at the 5 th day of life These data correspond to the absence of eosinophile cell infiltrations in infants of diabetic mothers with a longer duration of life Therefore On the fourth to fifth neonatal day the hyperactive appearance of the beta cell nuclei and the eosinophilia have disappeared and the k value has decreased to normal This time relation suggests a causal relation between eosinophilia and hyperinsulinism

Mckay Benirschke & Curtis (1953) suggested that some unknown substance from the hypertrophied islet possibly related to insulin diffuses into connective tissue and attracts the eosinophils leading to the peri insular cell infiltrations Recently *Phil Gustafson Josefsson & Paul* (1967) demonstrated that granula of eosinophile leucocytes contain zinc a substance which is found in the beta cells of the pancreas and to which insulin has a great affinity This finding may also suggest a relation between islet hypertrophy hyperinsulinism and the eosinophile cell infiltrations

A strong argument against this relation has been the absence of eosinophile cells in the pancreas of infants with erythroblastosis foetalis and islet hypertrophy Recently, *Steinke & Driscoll* (1967) demonstrated a high pancreatic insulin content in infants with severe erythroblastosis foetalis later (*Steinke Gries & Driscoll* 1967) they explained this phenomenon as a compensatory reaction due to binding of insulin to haemoglobin as well as to destruction of it by glutathione liberated from red blood cells In accordance with this newborn infants with rhesus immunization have normal disappearance rates of glucose (k values) after an intravenous glucose load (*Euler Larsson & Persson* 1964) indicating an absence of or a very small degree of hyperinsulism in these infants

Antibodies to exogenous insulin which are transfered via the placenta from the mother (*Spellacy & Goetz* 1963) do not play any role in a formation of the eosinophile infiltrates since in *Silverman*s

(1963) as well as in the present study marked eosinophilia was found in infants of diabetic mothers who had never received exogenous insulin

The occurrence of oedema or fibrosis and other inflammatory cells within the eosinophile cell infiltrates in the pancreas of infants of diabetic mothers has been described (*D Agostino & Bahn* 1963) the infiltrate was classified as a subacute inflammation By intraperitoneal or intravenous injection of guinea pig antiinsulin serum into rats *Lacy & Wright* (1965) caused similar morphological changes to develop in the exocrine portion of pancreas Their findings included marked oedema leucocyte infiltration consisting predominantly of eosinophiles and mononuclear cells and occasionally haemorrhage and necrosis This observation fits the general characteristics of allergic inflammation In the present material however neither oedema nor cells other than eosinophiles were present to any extent and interstitial fibrosis was not found Therefore an allergic origin of the eosinophiles is less likely

The increased haemopoiesis in the pancreas of infants of diabetic mothers demonstrates an immature pattern as does the higher incidence of lymphocyte infiltration (Table 2) Usually infants of diabetic mothers are born some weeks before the calculated term but several factors *e g* the hyper bilirubinaemia in the neonatal period (*Olsen, Osler & Pedersen* 1963) the lack of development of ossification centres (*Pedersen & Osler* 1958) and the increased haemopoiesis in the liver (*Cardell* 1953) suggest that they functionally are even more immature than their gestational age would indicate Our finding of an immature pattern of pancreatic extramedullary haematopoiesis is in accord with this concept of physiological immaturity of infants of diabetic mothers

Several of the cells in the haemopoietic infiltrates may be eosinophile myelocytes (Fig 2) From *Silverman*s study (1963) and from the present results (Table 1 and 3) however it is obvious that eosinophile (Fig 1) and haemopoietic (Fig 2) cell infiltrations are of different origin

Our findings concerning the positive correlation between birth weight and islet hypertrophy and the lacking correlation between eosinophile infiltrates and the severity of maternal diabetes or parity of the mother are in accordance with previous findings (*Cardell* 1953a *Driscoll Benirschke & Curtis* 1960 *Silverman* 1963)

The previously mentioned cells which were described as degenerated eosinophiles could be macrophages phagocyting eosinophile material but the nuclear form makes it more probable that they are degenerated eosinophile granulocytes Furthermore it is known that Charcot Leyden crystals may arise from eosinophile cells by degeneration (*Ayres & Starkey* 1950) It seems obvious to suggest that infants with only degenerated eosinophiles and crystals around the hypertrophied

islets may have had "ordinary" eosinophile infiltrates at the time of birth indicating a simultaneous disappearance of the eosinophile cells and the hyperinsulinism. The neonatal changes in the beta cell nuclear size and in the glucose disappearance rate (k values) support this theory. The conformity in timing with respect to morphology and physiology and the positive relation between the degree of islet hypertrophy and eosinophile infiltrations makes the relation between hyperinsulinism and eosinophile cell infiltrations very likely. Since the final mechanism of the origin of the eosinophile infiltrates is unknown at this time, further studies of a possible relation between eosinophiles and the hyperinsulinism might be valuable.

SUMMARY

The pancreases of 48 newborn infants of diabetic mothers were studied with regard to the occurrence of different cell infiltrations and the grade of islet hypertrophy. Twenty infants with erythroblastosis foetalis and 62 infants of mothers without diabetes or rhesus immunization during pregnancy served as controls.

Three types of cell infiltrations could be separated, consisting mainly of eosinophile cells or lymphocytes or haemopoietic cells.

In infants of diabetic mothers the degree of haemopoiesis and infiltrates of lymphocytes indicated that these infants were more immature than their gestational age would indicate.

Eosinophile cell infiltrations only occurred in the diabetic group in combination with islet hypertrophy.

A positive correlation between grade of islet hypertrophy and occurrence of eosinophile cell infiltrations was demonstrated.

Charcot-Leyden crystals were found in all pancreases with higher grades of eosinophilia. Transitional cells interpreted as eosinophiles in degeneration were found.

These degenerated eosinophiles were also observed in a group of infants of diabetic mothers with pancreatic islet hypertrophy but without "ordinary" eosinophiles. The mean duration of life was short (10 hours) in the infants with "ordinary" eosinophile infiltrates and longer (5 days) in those with only degenerated eosinophiles.

Neonatal changes in nuclear size of the beta cells are in agreement with neonatal changes in the disappearance rate of glucose (k value) following an intravenous glucose load. The signs of activity of the beta cell nuclei and the k value express the hyperinsulinism. The disappearance of eosinophile cells corresponds in timing with the disappearance of the hyperinsulinism. It is suggested that the eosinophilia is causally related to hyperinsulinism.

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TRANSFER AMYLOIDOSIS

Local and Systemic Amyloidosis in Recipients of Syngeneic Spleen Grafts from Non Amyloidotic Casein Sensitized Donor Mice

By

FINN HARDT and POUL RANLOV

Received 11 i 68

Observations of the possible transfer of casein induced amyloidosis between syngeneic mice by means of intravenous injections of spleen cell suspensions or homogenates hereof derived from donors pretreated with 17 daily casein injections have earlier been published from this laboratory (*Werdelin & Ranlov* 1966 *Ranlov & Werdelin* 1967 *Ranlov* 1967) In these experiments such treated recipients developed spleen amyloidosis within 2–4 days following the transfer The recipients amyloidosis in this system depended on the previous hyperimmunization of the donors with at least 17 injections of casein

However this treatment apparently caused minute amounts of amyloid substance to appear in about ⅓ of the donor spleens with a consequent contamination of the pooled spleen cell suspension with amyloid material Immediately after the transfer this substance could be found as amyloid emboli in the recipient lungs In the course of the following two days spleen amyloidosis developed

To avoid this contamination of the recipient tissues with preformed amyloid of donor origin was one of the aims of the present study This purpose was served by the transplantation in a somewhat similar transfer system of splenic tissues from mice pretreated with 10 daily casein injections only Hence the grafted tissues contained hyper immunized cells but as yet no amyloid substance Subsequently the immunization was continued in the recipients with additional daily injections of casein for 10 days

While the earlier published transfer experiments employing spleen cells in suspension intravenously administered left few opportunities to study the morphological relationships between recipient amyloid and tissues (cells) of donor origin the present experiment was de

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signed in order to keep the donor cells as a morphological entity within the host

MATERIAL AND METHODS

The animals were randomly selected mice from our inbred colony of the C3H strain Sex distribution was equal all mice were between 8 and 10 weeks of age at the beginning of the experiment weighing from 23 to 27 Gm They were fed on oats

Donors 10 mice were given a total of 10 daily injections of ½ ml of a 5 per cent solution of sodium caseinate subcutaneously In addition 5 untreated control donors were included

Transplantation Under anaesthesia (Avertin®) two small slices (2 × 3 × 2 mm) of spleen were removed from each donor and immediately transferred to the lateral margins of the right kidneys of two anaesthetized recipient mice according to the method described by *Wheeler et al* (1966) Thus the 10 casein treated donor mice supplied spleen grafts for a total of 20 recipients These recipient mice are in the following referred to as the experimental group The remaining part of the donor spleens was kept for histological examination Similarly 5 not pretreated control donors supplied spleen grafts for a total of 10 recipients which in the following are referred to as the control group Likewise the remainder of the control donor spleens were kept for histological evaluation

Recipients The recipients recovered rapidly after the transplantation No deaths occurred during the operation but two recipients—one from the experimental and one from the control group—died two days later from haemorrhage These were excluded The day after the transplantation the first of a series of 10 daily injections of ½ ml of 5 per cent sodium caseinate was given This treatment was administered both to the experimental and the control group On the day of the last casein injection the first of two injections of 0 05 mg of nitrogen mustard (Erasol®) in ½ ml of saline was given subcutaneously to both groups The second injection was given two days later Nitrogen mustard was applied in order to accelerate the presumed amyloid formation (*Teilum* 1954) The day after the last nitrogen mustard injection that is on day 14 after the transplantation all recipients were killed

The right kidney carrying the spleen graft was fixed in neutral formalin and embedded in paraffin separately Left kidney liver lung and spleen were likewise fixed and embedded Section were cut 5 microns thick and stained with haematoxylin eosin methyl green pyronin alkaline Congo red and the PAS stain Amyloid was identified by its morphology and by its birefringence with Congo red under crossed polars

The degrees of amyloidosis if any were evaluated on section of donor spleens spleen grafts and host spleen according to the semiquantitative method ranging from 0 to 6 described by *Christensen & Hjort* (1959)

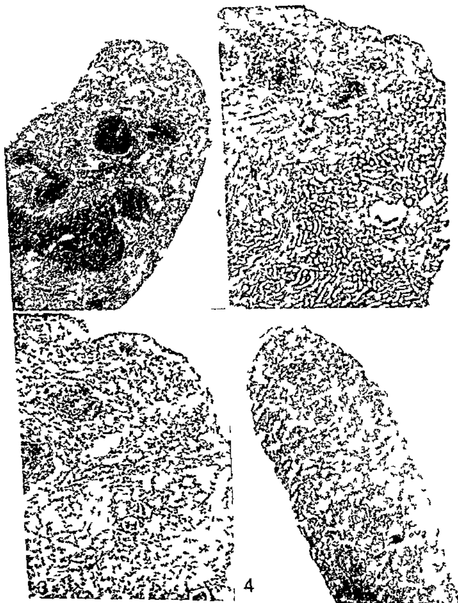
Figs 1–5

Fig 1 Donor spleen from untreated control donor at the time of transfer Alkaline Congo red × 56

Fig 2 Spleen graft from untreated donor spleen shown in Fig 1 placed on the lateral edge of the right kidney of a control recipient After grafting the recipient was treated with 10 daily injections of casein followed by a short course of HN2 The structure of the spleen graft seem well preserved and no amyloid is detectable Alkaline Congo red × 56

Fig 3 Larger magnification of spleen graft shown in Fig 2 No demarcation zone between graft and adjoining host kidney No visible amyloid Alkaline Congo red × 140

Fig 5 Spleen from the same recipient control as shown in Figs 2 and 3 That is following grafting treated with 10 days of casein and 2 HN2 injections Amyloidosis could not be found Alkaline Congo red × 56



4

RESULTS

The main results of the present investigations are outlined in Table 1. From the histological examinations of the donor spleens at the time of grafting, it appeared that amyloid could not be found in any of the spleens from the untreated control donors (Fig. 1) and that the same was found to be the case in 6 out of 10 spleens from casein pretreated donor mice, while the remaining 4 spleens in the experimental donor group exhibited such minor amounts of amyloid material as to be hardly detectable.

In the control group of recipients which had received spleen grafts from untreated donors, followed by 10 days of casein and a short course of HN2, amyloidosis did not develop neither in the graft (Figs. 2 and 3) nor in the host tissues (Fig. 4). In contrast, the experimental group in which animals had received spleen grafts from donors pretreated with casein for 10 days, the grafting being followed by additional 10 casein injections and a short course of HN2, all showed a severe and widespread amyloidosis, especially pronounced in the graft and in the host spleen, the latter often to the maximum degree (Figs. 5–8 and 9–12). It further appears that a correlation between the degree of amyloidosis in the graft and in the host spleen exists (Table 1).

The systemic amyloidosis in the mice in the experimental group showed the usual pattern of distribution: the amyloid was most abundant in the spleen (Figs. 8 and 10), to a lesser extent in the liver, and only sparse in the kidneys. However, this pattern showed one exception: rather heavy amyloid infiltration was evident in the kidney tissue immediately adjacent to the amyloidotic spleen graft, in contrast to the minor involvement of the rest of the recipient kidney and the contralateral kidney of the same animal. This local amyloid infiltration involved the tubular interstitial tissues as well as the glomeruli (Figs. 6, 7, 9, 11, 12).

Figs. 5–8

Fig. 5. Donor spleen after 10 days of pretreatment with casein—at the day of grafting (donor F, 3 in Table 1). The spleen shows increased cellularity and perifollicular proliferation and transformation but as yet no visible amyloid. Alkaline Congo red × 56.

Fig. 6. Right kidney of recipient (3B in Table 1) carrying spleen graft from the donor spleen shown in Fig. 5. After additional 10 days of casein treatment in the recipient and a short course of HN2, the graft shows massive amyloid deposition in contrast to the apparently amyloid free adjacent host tissues. Alkaline Congo red × 56.

Fig. 7. Larger magnification of spleen graft shown in Fig. 6. The amyloid substance of the spleen graft is seen extending into the adjacent host kidney, involving the tubular interstitial tissues and a few glomeruli. In contrast, no significant amyloid could be demonstrated in the remaining part of this or the contralateral kidney. Haematoxylin eosin × 140.

Fig. 8. Spleen from the recipient of Fig. 7, showing severe systemic amyloidosis. Haematoxylin eosin × 56.

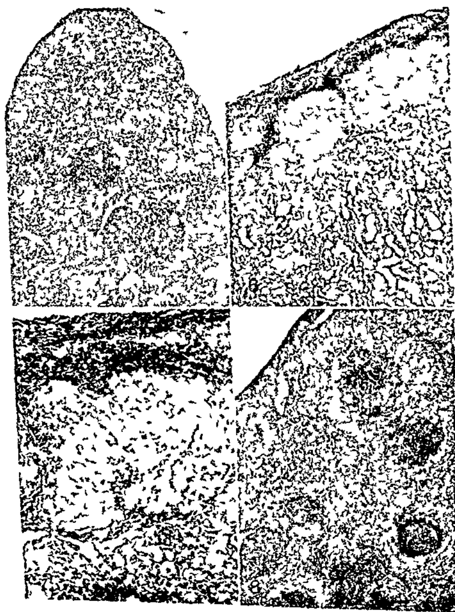


TABLE 1

Experimental Design and Results

Donors			Recipients				
No	Pretreatment	Donor spleen amyloid	No	Treatment of recipients	Amyloid in graft	Host spleen amyloid	Host liver amyloid
Co 1	None	0	1a	Casein × 10	0	0	0
			1b	†	†	†	†
Co 2	None	0	2a	Casein × 10	0	0	0
			2b	Casein × 10	0	0	0
Co 3	None	0	3a	Casein × 10	0	0	0
			3b	Casein × 10	§	0	0
Co 4	None	0	4a	Casein × 10	§	0	0
			4b	Casein × 10	§	0	0
Co 5	None	0	5a	Casein × 10	0	0	0
			5b	Casein × 10	0	0	0
Ex 1	Casein × 10	1	1A	Casein × 10	5	5	+
			1B	Casein × 10	4	6	+
Ex 2	Casein × 10	1	2A	Casein × 10	5	5	+
			2B	Casein × 10	5	5	+
Ex 3	Casein × 10	0	3A	Casein × 10	4	5	+
			3B	Casein × 10	§	§	+
Ex 4	Casein × 10	0	4A	Casein × 10	4	6	+
			4B	Casein × 10	4	5	+
Ex 5	Casein × 10	0	5A	Casein × 10	5	6	+
			5B	Casein × 10	§	5	+
Ex 6	Casein × 10	0	6A	Casein × 10	§	6	+
			6B	Casein × 10	4	5	+
Ex 7	Casein × 10	0	7A	Casein × 10	4	5	+
			7B	Casein × 10	4	5	+
Ex 8	Casein × 10	2	8A	Casein × 10	5	5	+
			8B	†	†	†	†
Ex 9	Casein × 10	1	9A	Casein × 10	5		+
			9B	Casein × 10	4	5	+
Ex 10	Casein × 10	0	10A	Casein × 10	3	4	+
			10B	Casein × 10	4	4	+

(† = died during experiment) (§ = tissues lost during preparation)

Figs 9-12

Fig 9 Spleen graft from casein treated donor grown on the right kidney of a recipient subsequently treated for 10 days with casein Excessive amyloid in graft Alkaline Congo red × 56

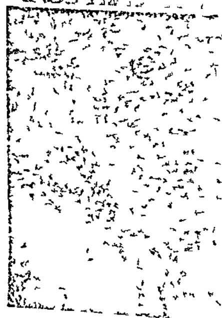
Fig 10 Host spleen from the recipient of Fig 9 Marked systemic amyloidosis Alkaline Congo red × 56

Fig 11 Larger magnification of the section shown in Fig 9 Infiltration of amyloid substance into the adjacent host kidney Alkaline Congo red × 140

Fig 12 Exactly the field of section as shown in Fig 11 This picture, however, has been taken through crossed polar The amyloid substance in the graft as well as the amyloid infiltrating the host kidney shows vivid green birefringence Alkaline Congo red, crossed polar × 140


9


10


11


12

The findings support earlier results indicating the existence of an inducer or trigger substance responsible for the initiation of amyloid formation from reticulo endothelial cells in hyperimmunized animals

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THE CONNEXION BETWEEN MYOCARDIAL INFARCTION AND GALLSTONES IN AN AUTOPSY SERIES

By

F BERGMAN W VAN DER LINDEN and J SÖDERSTRÖM

Received 5 ii 68

The information regarding the relationship between ischaemic heart disease and gallstone disease is of a very ambiguous nature On the one hand there is considerable evidence inter alia provided by studies of geographical pathology which indicate an association between the two diseases On the other hand there are a number of characteristics for instance their highly divergent sex ratios which are definitely indicative of an inverse relationship In the present paper the connexion between the two diseases is studied in an autopsy series In this study special attention is paid to problems of selection

MATERIAL AND METHOD

The present investigation is based on the autopsies performed during the five year period 1957 1961 at the *Dept of Pathology* in the *Malmo General Hospital*

TABLE 1
Number of Cases of Recent and Healed Myocardial Infarction in Different Sex and Age Groups

	Recent infarction		Healed infarction	
	♂	♀	♂	♀
40 – 49	16	2	11	2
50 59	55	12	59	16
60 69	107	50	134	69
Σ	178	64	197	87

Four groups consisting of male and female patients with recent and healed myocardial infarction were studied *Recent* infarction was considered present not only in cases with distinct gross or microscopical signs of fresh myocardial necrosis but also in cases where the coronary arteries were occluded by a recent thrombosis although distinct histological changes were not yet discernible Cases with well outlined fibrous foci in the myocardium were classed as *healed* infarctions. Cases with both recent and healed infarctions were classed as recent only Table 1 gives

the number of recent and healed infarctions in different sex and age groups For each case of infarction in patients between 40–69 years of age the next case in the autopsy files of the same sex and the same age and without myocardial changes was taken as a control In that way four infarction groups were formed consisting of men and women with recent and healed infarctions respectively These four groups were compared with four control groups in which the age distribution was similar In the highest age groups (70 and older) the scarcity of cases without myocardial changes and thus suitable as controls made it necessary to limit the study to include only patients in the age group 40–69 years

The incidence of cases of gallstones and cases on which cholecystectomy had been performed was noted and so was the incidence of cases in which the gallbladder showed macroscopical or microscopical signs of acute or chronic cholecystitis Comparisons between the four infarction groups and the four control groups were made with a view to the occurrence of such lesions

Statistical significance was tested with the χ^2 test with Yates correction

RESULTS

Although the total series included about equal numbers of male and female patients it was found in the five year period of investigation that the incidence of recent myocardial infarction was twice as high in male as in female patients Healed infarction was also found far more often in the males Table 1 gives the age and sex distribution of the infarction cases

TABLE 2

Patients with Gallstones and Cholecystectomized Patients in the Infarction and Control Groups

	Males			Females		
	+	—	Σ	+	—	Σ
Recent infarction	44 (6)	134	178	35 (8)	29	64
Control	41 (7)	137	178	28 (10)	36	64
P	> 0 05			> 0 05		
Healed infarction	57 (10)	140	197	57 (6)	30	87
Control	53 (11)	144	197	39 (6)	48	87
P	> 0 05			< 0 01		

+ denotes gallstones present or patient cholecystectomized

— denotes absence of gallstones and patient not cholecystectomized

The figures within brackets denote the number of cholecystectomized cases

Table 2 gives the number of patients with gallstones or patients who had been cholecystectomized in the four infarction groups and in the control groups Such patients were found more often in the infarction groups than among the controls However the difference was significant only when females with healed infarction and their controls were compared

Finally Table 3 gives the number of cases with signs of acute or chronic cholecystitis As seen in this Table cholecystitis was found less often in the gallstone cases in the four infarction groups than in the gallstone cases in their respective control groups When the oc

currence of cholecystitis in gallstone patients with recent infarction was compared with that in gallstone patients in the control group a significant difference was found Cholecystitis appeared to occur less often in the former groups

TABLE 3

Occurrence of Cholecystitis in Cases of Gallstone in Infarction Groups and Controls

	Males			Females		
	+	–	Σ	+	–	Σ
Recent infarction	4	34	38	7	20	27
Control	18	16	34	11	7	18
P		< 0 001			0 05 > P > 0 01	
Healed infarction	12	35	47	11	40	51
Control	18	29	47	12	21	33
P		> 0 05			> 0 05	

+ denotes signs of acute or chronic cholecystitis
– denotes absence of such signs

DISCUSSION

In the study of gallstone disease as well as in that of ischaemic heart disease a number of features have been found which seem to point to a link between the two In the first place there is the suspicion of a disturbance of the cholesterol metabolism which is common to both Furthermore both diseases prevail in the populations belonging to the Western cultural pattern and are seldom found in the so called under developed areas Another indication of a connexion is perhaps the rise in incidence of both diseases as observed in Scandinavia as well as in many other countries during the years following World War II

On the other hand a number of epidemiological and other characteristics seem to point to an inverse relationship This is indicated inter alia by the results of studies of certain tribes of North American Indians In these people who seem to have rather low serum cholesterol levels (Abraham & Miller 1959) gallstone disease is very common (Hesse 1959) while myocardial infarction is a rare occurrence (Fulmer & Roberts 1963) The sex ratio a prevalence of men among patients with myocardial infarction and of women among the gallstone patients also points to an inverse relationship

In view of this divergency we thought it of interest to study the relationship of the two diseases in an autopsy series

In the present series there were more than twice as many male as female infarction cases This held true whether recent or healed infarction were considered On the other hand both in the infarction groups and in the control groups gallstones were found to occur more often among the females These two findings are of course in agreement with the well known sex ratios of these two diseases

In the present analysis, cholecystectomized cases were considered to have suffered from gallstones prior to operation. We feel this is justified as cholecystectomy is only very rarely performed in cases without gallstones. In the present autopsy series gallstones were found to occur more often in cases of myocardial infarction than in cases without. This held true for all the studied groups, though the level of significance was reached only when female patients with healed infarction were compared with their matched controls. *Mainland* (1953) has pointed out that if two disease groups in a necropsy series are compared regarding the frequency of the occurrence of a third disease, a spurious association between the third disease and the disease group with lower fatality rate arises. This bias, for an algebraic analysis of which the reader is referred to *van der Linden* (1961), complicates studies of the association of diseases on the basis of autopsy series. It cannot, however, explain the association found here. In the comparison between infarction cases and controls, the former, in which gallstones were found to occur more frequently, have not, of course, a lower fatality rate than that of the controls. Consequently the positive association cannot be a statistical artefact. This finding of an association between gallstones and ischaemic heart disease is in agreement with the reports of *Zschoch* (1966) and *Kozoll, Dwyer & Meyer* (1959).

When the gallstone cases in the four infarction groups were compared with those in the control groups (see Table 2) little difference in the number of cholecystectomized cases was found. On the other hand, as seen in Table 3, there was a marked difference in the occurrence of cases with signs of cholecystitis. This difference, which was apparent by all the four comparisons, reached the level of significance when the two groups of recent infarction and their respective controls were compared. Signs of cholecystitis were found less frequently in gallstone patients with acute infarction. In our opinion this finding is to be regarded as a statistical artefact. In a comparison between two groups, cases with recent infarction and cases without signs of infarction, in which the fatality rate may be assumed to be highly different, the frequency of occurrence of a third disease, cholecystitis, is found to be lower in the group with the highest fatality rate. It is of interest to note that the negative association cholecystitis/infarction, which we believe to be due to statistical bias, is significant only in the cases of recent infarction. These cases may be assumed to differ considerably from their controls as regards fatality rate. On the other hand, the positive association gallstone/infarction, which we believe exists in spite of the same bias, is significant only in the female cases of healed infarction. The difference in fatality rate between healed infarction cases and controls is probably not so large.

Already at the beginning of this century (*Babcock* 1909) a causal link between gallstone disease and ischaemic heart disease, in the sense that the mere existence of the former could have a noxious effect on

the heart was suspected Clinical observations of relief of cardiac symptoms after cholecystectomy (*Walters & Master* 1952) and of electrocardiographic changes during biliary distention (*Hodge & Messer* 1948) supported this belief However the association between gall stones and ischaemic heart disease found in this autopsy series should not be interpreted to the effect that it reflects such a causal link

In the infarction groups cases of cholecystitis were fewer Even if this finding were considered a statistical artefact the opposite would have been the case if the association gallstones/ischaemic heart disease were due to a deleterious influence of gallstone disease on the heart Therefore we are inclined to agree with *Zschoch* (1966) who regards the association gallstones/ischaemic heart disease as a possible expression of some common pathogenetic principle Such a view would be in accordance with the results obtained in recent studies on experimental gallstone formation (*Tepperman Caldwell & Tepperman* 1964 *Bergman & van der Linden* 1967) which indicate a parallelism between gallstone formation and experimental atherosclerosis

SUMMARY

In an autopsy series cases with recent and healed myocardial infarction were compared with sex and age matched controls without myocardial changes Gallstones were found to occur more often in the infarction cases than in the controls This association was considered genuine On the other hand signs of cholecystitis were found less often in the infarction cases than in the controls but this negative association was considered spurious The findings are discussed and reasons are given why the association gallstones/infarction is not considered to be due to a noxious effect of gallstone disease on the heart

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LOCALIZATION OF α FOETOPROTEIN IN THE HUMAN FOETUS AND PLACENTA

By

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Received 7 xii 67

The occurrence of a specific serum protein α foetoprotein in the human foetus was described by *Bergstrand* in 1956 (1) and this observation has been confirmed by a number of authors

The site of synthesis is not known but the rapid decrease in serum level of this protein *post partum* indicates that the placenta might be responsible for the synthesis (2 8)

To investigate the role of the placenta in this respect specific anti serum against α foetoprotein was used for immunofluorescent local ization

MATERIAL AND METHODS

Antiserum The preparation of specific antiserum against human α foetoprotein has been described previously (7) Sheep were used for immunization

Antigen Foetuses of 6 to 12 weeks gestation were studied Legal abortions were performed by caesarean section and the foetuses were obtained intact together with the placenta and membranes Placenta liver and kidney were cut into small blocks snap frozen and freeze dried After embedding in paraffin wax m p 56 C the blocks were sectioned at 2–5 μ in a serial microtome dewaxed in xylene passed through absolute alcohol and dried

Fluorescent tracing was performed using the indirect method of Coon Gamma globulin electrophoretically separated from sheep serum was used to immunize rabbits The resulting antiserum was fractionated with Na_2SO_4 (4) to yield gamma globulin which was labelled by the method of *Lewis et al* (5) Fluorescein isothio cyanate (FITCH) isomer I was provided by Baltimore Biological Corp A Wild fluorescence microscope was used to examine the slides

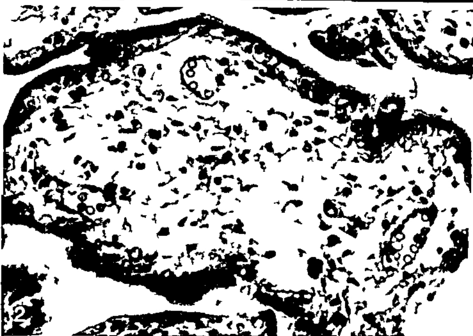
Staining Procedure

1st step Premoistened sections were incubated for one hour at room temperature with undiluted sheep anti-α foetoprotein serum (AF) then rinsed in phosphate buffered saline (PBS) pH 7 1 for 15 min under constant agitation

2nd step Sections were incubated with fluorescent anti sheep gamma globulin

This work was supported by grants from the President J K Paasikivi Foundation the Yrjö Jahnsson Foundation and the Finnish Medical Research Council


1


2

(FAShγ) protein concentration 0 02 per cent for 30 min at room temperature then rinsed in PBS for one hour

Controls

A number of controls were used to evaluate the specificity of the observed fluorescence These included treatment of the tissue sections with 1 free FITCH 0 2 mg/ml in PBS 2 FAShγ only 3 AF absorbed with lyophilized foetal serum 100 mg/ml compared with AF absorbed with the same amount of lyophilized normal adult human serum 4 Labelled AF for direct staining

RESULTS

Placental Localization

In cross sections of placental villi the fluorescence was weak and appeared as irregular circles (Fig 1 2) The circles corresponded to the walls of the foetal capillaries There was no fluorescence in the villar stroma or the trophoblast cells

Liver Localization

Fluorescence in the liver was confined to the cytoplasm of the hepatic cells (*Fig 3 4*) In the haemopoietic cells which appeared in clusters there was no fluorescence Interestingly the periportal regions showed a very intense staining Inside the vessels there was a faint staining of the erythrocytes

Kidney Localization

In the kidney there was a distinct fluorescence in the mesenchyme located between the structural elements (Fig 5 6) The loose mesenchyme surrounding the collecting tubules in the medullary region had a particularly strong fluorescence There was no fluorescence in the tubular cells and the glomerular fluorescence was insignificant The specificity of the observed fluorescence in placenta liver and kidney was indicated by the lack of staining when FAShγ alone or AF absorbed with foetal serum was used On the other hand the ability to react was not influenced by absorption of AF with normal adult serum Free fluorescein gave a generalized staining of all organs studied Direct staining with labelled AF gave a weak fluorescence in the liver and the kidney corresponding to that observed using the indirect technique This fluorescence was significantly weakened by blocking with unconjugated antiserum

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Figs 1–2

Fig 1 Localization of α foetoprotein in the placenta Cross section of villus is seen Weak fluorescence confined to walls of foetal capillaries Note absence of fluorescence in trophoblast cells × 300

Fig 2 Haematoxylin and eosin (H E) stained section of villus showing foetal capillaries × 300


3
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DISCUSSION

The present study localized α foetoprotein to the hepatocytes of human foetal liver This finding correlates well with the recent work of *Gitlin & Boesman* (3) who showed that incorporation of labelled amino acids into α foetoprotein occurs in foetal liver tissue *in vitro* The foetal vessels were the only part of the placenta that was stained This indicates that α foetoprotein is not synthesized by the placenta The faint staining of the vessel walls may be attributed to diffusion from the foetal circulation Gitlin and Boesman observed no incorporation either by placental tissue or kidney tissue The observed incorporation by rat yolk sac membrane is very interesting and may explain the decreased synthesis after birth

The localization in the kidney was identical with that obtained by antisera against foetal kidney absorbed with normal adult serum plus normal adult kidney (6)

The fluorescence in the periportal mesenchyme is apparently analogous to that observed in the mesenchyme of the kidney The question whether this mesenchymal fluorescence represents sites of active synthesis cannot be answered by localization studies The absence of fluorescence in the villar stroma is interesting in this respect It raises the question whether the discrepancy is due to differences in the barrier between circulation and tissue or in the properties of the connective tissues

SUMMARY

The localization of the foetus specific protein α foetoprotein in the placenta liver and kidney of human foetuses was studied by immunofluorescent staining In the placenta only the foetal vessels were stained In the liver α foetoprotein was found in the hepatocytes and periportal mesenchyme In the kidney the mesenchyme was distinctly stained

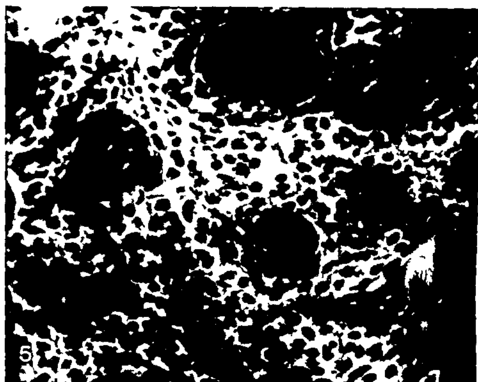
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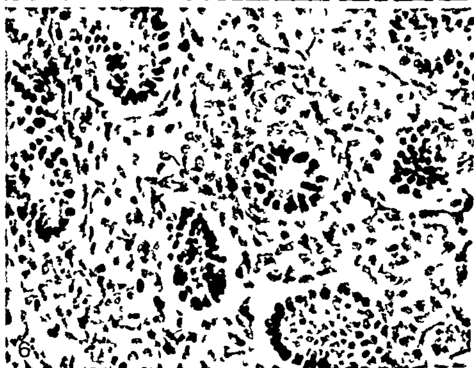
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Figs 3 4

Fig 3 Localization of α foetoprotein in foetal liver Staining of hepatocytes and periportal mesenchyme Red blood cells in the lumen of a portal vessel are weakly stained Clusters of unstained haemopoietic cells are n × 450

Fig 4 H E stained section parallel to that of Fig 3 No portal vessel lumen is seen in this section Periportal mesenchyme is on the left Note clusters of haemopoietic cells × 450


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6

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Figs 5–6

Fig 5 Localization of α foetoprotein in foetal kidney Bright staining of mesenchyme located between tubules in the cortical region × 90
Fig 6 H E stained section of the same region as in Fig 5 showing loose mesenchyme between tubules and primitive glomeruli × 90

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DEVELOPMENT OF STRAINS OF *CRYPTOCOCCUS NEOFORMANS* RESISTANT TO NYSTATIN, AMPHOTERICIN B, TRICHOMYCIN AND POLYMYXIN B

By

JESSIE BODENHOFF

Received 25/1 68

The literature contains very few references to investigations into the development of resistance by fungi to antimycotic drugs (*Casida & McCoy* 1951 *Donovick et al* 1955 *Drouhet* 1955 *Stout & Pagano* 1956 *Bradley* 1958 *Littman et al* 1958 *Jones & Peacock* 1959 *Sorensen et al* 1959 *Hebeka & Solotorovsky* 1962 1965) Some of these reports are concerned with the development of resistance by *Candida albicans* and other strains of Candida By contrast induced resistance in *Crypto coccus neoformans* has not been described It would therefore seem justified to report some experiments designed to produce resistance in certain strains of *Cryptococcus neoformans* to nystatin amphotericin B trichomycin and polymyxin B

MATERIAL AND METHODS

Strains

Five strains of *Crypto occus neoformans* have been used in the experiments M 14–372 (*Rieu erts Friksen & Frichsen* 19[illegible]) originating from pigeon droppings[1] CBS 132 and CBS 131 received from Centraalbureau voor Schimmelcultures Yeast Division Delft A 314 and A 310 isolated from samples of cerebrospinal fluid sent for investigation in the mycological laboratory Statens Seruminstitut[2] The clinical history of one of these patients has been published previously (*Risgaard Petersen* 19[illegible])

None of the strains investigated had previously been in contact with antimycotic agent

Inoculi

Crypt[illegible]s neoformans cultured on Sabouraud s maltose agar (pH = [illegible]) for 3 days at 3[illegible] as suspended in sterile saline to give an optical density corresponding to a cell [illegible] per ml in the region of [illegible]–8 × 10^6 0 1 ml of this suspension was used as inoculum [illegible] inoculum)

My thanks are due to *Knud Rieu erts Friksen*[1] M D Lecturer and Head of Department Institute of Medical Microbiology University of Copenhagen and Dr *Per Holm*[2] Head of Dept., Statens Seruminstitut who have supplied me with these strain

Determinations of Sensitivity

The sensitivity of the strains of Cryptococcus was determined by the plate dilution method Sabouraud's maltose agar (pH = 6) with increasing amounts of antimycotic was used as substrate Each plate contained 30 ml of substrate Culture was carried out at 37 C for five days Two values were recorded as expressions of sensitivity

(1) Min IC (= minimal inhibitory concentration) i e the least concentration of antimycotic producing complete inhibition and
(2) Max GC (= maximal growth concentration) which was the maximum concentration of antimycotic in which obvious growth could be seen over the whole plate

This latter has been used in the calculation of the index of increase in resistance i e the ratio between the sensitivity of the resistant strain to any antimycotic and the sensitivity of the original strain The author considers that the ratio between Max. GC gives a more accurate impression of the index than the ratio between Min IC

Induction of Resistance

Szybalski's gradient plate technique (1952) has been used to induce resistance Sabouraud's maltose agar was used for the Szybalski plates The slanting bottom layer consisted of 15 ml of agar without antimycotic The top layer consisted of 15 ml of agar to which antimycotic had been added in the appropriate concentration The suspensions were spread with equal density over the entire surface The plates were incubated at 37 C, for three days on first passage and for five days on later passages For each experiment several Szybalski plates with increasing concentrations of antimycotic in the top layer were spread Culture from the plate containing the highest concentration of antimycotic which showed obvious growth was used as material for the next experiment in which gradient plates containing higher concentrations of antimycotic were used Taking as example strain 4 with amphotericin B as antimycotic the following concentrations were used in the top layer of the Szybalski plates 0 5 1 0 2 5 5 10 15 25 50 75 100 250 500 750 and 1000 µg/ml of substrate Corresponding concentrations were used in all gradient plate experiments During the experimental period sensitivity determinations were carried out as frequently as was practical possible At the conclusion of the experiment all five strains were investigated for induced restistance by comparison with the original sensitive strain after culture on resistance plates All the strains both original and resistant have been stored at −25 C since the conclusion of the experiment

Antimycotic Agents

The following procedures were employed for the production of solutions of the antimycotics

Nystatin (Mycostatin® Squibb) 10 mg of nystatin was weighed in a sterile flask It was rubbed to a paste with 1 ml of 70 per cent ethanol by means of a sterile glass spatula and thereafter allowed to stand in a refrigerator for 2 hours 9 ml of sterile water were added and the suspension thoroughly shaken The concentration in the suspension was of the order of 1000 µg nystatin per 1 ml suspension

Amphotericin B (Fungizone® Squibb) 50 mg of amphotericin B (in complex combination with sodium desoxycholate) was weighed in a sterile flask and dissolved by the addition of 10 ml of sterile distilled water

Trichomycin (Astra) 5 mg of trichomycin was weighed in a sterile flask and dissolved in 17 5 ml of 0 01 N sodium hydroxide If the solution was not completely clear one or two drops of 4 per cent sodium hydroxide were added

Polymyxin B (Pfizer) 1 g of polymyxin B sulphate was weighed and dissolved in 100 ml of sterile water

In a pilot study it was demonstrated that neither sodium hydroxide nor alcohol had any fungistatic or fungicidal effect in the concentrations used

Demonstration of Cross Resistance

Nystatin resistant cryptococcal cells developed from *Cryptococcus neoformans* strains 3 4 and 5 were studied in experiments designed to reveal cross resistance to

TABLE 3

Colony Diameter in Original Strain (Strain 2) and in Maximally Amphotericin B Resistant and Polymyxin B Resistant Cryptococcus neoformans *(Strain 2)*

Original strain	Amphotericin B resistant strain	Polymyxin B resistant strain
2.52	1.34	0.17
2.58	0.28	0.14
2.35	1.65	0.11
2.80	0.20	0.08
2.24	0.22	0.17
2.18	0.20	0.14
2.24	0.22	0.14
2.35	2.02	0.11
2.02	0.03	0.03
1.96	0.17	0.11
2.13	0.27	0.14
2.46	0.59	0.17
2.52	0.17	0.14
2.13	0.11	0.11
2.46	0.25	0.11
2.35	1.09	0.11
2.13	2.04	0.14
2.58	0.70	0.14
2.13	0.95	0.08
2.18	0.59	0.17
2.18	1.57	0.17
2.24	1.65	0.17
2.35	1.65	0.08
2.30	0.34	0.17
2.30	0.22	
2.18		
2.18		
2.30		
2.18		
2.30		
2.24		
2.30		

Suspensions of five day old cultures of the sensitive strain (from Sabouraud's maltose agar) and resistant cryptococcal cells (from resistance plates with concentrations of 1000 µg of antimycotic/ml substrate) cultured on Sabouraud's maltose agar (pH = 5) at 3[illegible] C for three days. Colony diameter (stated in mm) measured by means of ocular micrometer in a low magnification microscope.

In these experiments designed to produce loss of polymyxin B resistance by the cryptococcal cells it was observed that after the many passages on substrate containing no antimycotic the size and shape of the colonies approached more closely that of the original sensitive strain despite the fact that as mentioned above they retained their resistance.

By contrast the amphotericin B resistant cryptococcal cells rapidly lost their resistance after being spread onto substrate which contained no antimycotic. They were originally so resistant that they were capable of growth on substrate containing 500 µg of amphotericin B/ml but after a few [illegible] passages on Sabouraud's maltose agar they were no longer able to grow [illegible] Sabouraud's agar containing amphotericin B



Fig 3

Partial dependence on antimycotic agents in polymyxin B resistant cryptococcal cells (Strain 2) on Sabouraud's maltose agar containing no antimycotic (left plate) and containing 50 µg of polymyxin B/ml substrate (right plate) Growth is obviously better on substrate containing antimycotic Culture at 37 C for nine days

in concentrations greater than 5 µg/ml The resistance of the amphotericin B resistant cryptococcal cells must be characterized as very labile

There was thus a very clear difference in the stability of the resistance developed in the polymyxin B resistant and amphotericin B resistant cryptococcal cells

Partial dependence on the antimycotic was observed in all five strains of cryptococcus one strain (Strain 2) exhibited dependence on all four antimycotics The partial dependence on the antimycotic was the more pronounced the more stable the induced resistance The dependence was not so marked in any of the resistant strains that they could not grow on substrate which did not contain antimycotic but after the addition of antimycotic in even very low concentrations there was an obvious stimulation of growth (Fig 3)

At high degrees of resistance the colonies seemed to be less pigmented the mucoid character was lost and the colonies seemed dull and dry Gram stained preparations revealed no differences between the staining of the sensitive and resistant cells Whilst the original sensitive cryptococcal cells were seen in tusch preparations to lie singly and to be surrounded by a capsule poor capsule formation was observed in one of the strains (Strain 2) namely the polymyxin B resistant strain in which the resistance to the antimycotic was most pronounced furthermore the cells in the polymyxin B resistant strain showed a tendency to formation of conglomerates

Biochemical Changes

The ability to ferment and assimilate carbohydrates and the ability to assimilate nitrogen were investigated in both original and resistant

cryptococcal cells No changes were observed in the biochemical reactions of the resistant cells other than those which could be explained by the reduced rate of growth in these strains

DISCUSSION

It is apparent from the experimental results that it is possible *in vitro* to produce resistance of *Cryptococcus neoformans* to nystatin amphotericin B trichomycin and polymyxin B

Whilst the resistance of bacteria to antibiotics is well known there are few reports in the literature of the development of resistance to antimycotic agents in fungi this is apparent from the reports mentioned in the introduction most of which are furthermore concerned with the development of resistance to amphotericin B and nystatin in species of *Candida*

The production of resistance to nystatin and amphotericin B was found to be much easier than was expected from the studies of the development of resistance in other members of the group of yeasts It must be presumed that the reason for this was the use of Szybalski's gradient plate technique as in this technique there are in practice no steps in concentration This is in accordance with *Littman et al* s (1958) observation that the use of small steps in concentration and many passages over longer periods of time facilitates the induction of resistance in Candida

Not all strains of cryptococcus exhibit the same facility in developing resistance to any given antimycotic this is again in accordance with the findings of *Littman et al* (1958) in species of Candida

A given strain of cryptococcus does not develop resistance to the different antimycotics with equal ease It is apparent from the results of the investigation that the induction of resistance to nystatin is complicated by the difficulties in producing a solution of nystatin it is necessary to work with a suspension the nystatin concentration of which is not exactly known Furthermore both nystatin and amphotericin B are labile compounds Where trichomycin is concerned it was occasionally found that it was not possible to obtain growth at the same concentration as in the preceding experiment and for this reason it was necessary to go backwards in the concentration series in order to obtain growth again This is the explanation of the relatively large number of passages which were used in the trichomycin experiments without the induction of maximal resistance to trichomycin

During the course of the experiments the author had the impression that [illegible] a certain degree of resistance had been induced it was possible to [illegible] a greater degree more rapidly this confirms the observations reported by *Sorensen et al* (1959)

During the pr[illegible] investigation cross resistance between nystatin and amphotericin B has been demonstrated in two of the three strains

studied (Strains 4 and 5) *Hebeka & Solotorovsky* (1965) were not able to demonstrate cross resistance to nystatin and amphotericin B in Candida albicans but they found such cross resistance between candidin and amphotericin B Cross resistance between the polyene antimycotics has been demonstrated in Candida albicans by *Littman et al* (1958) and *Sorensen et al* (1959)

As in *Hebeka & Solotorovsky s* (1965) investigations it has been possible to demonstrate changes in the morphology pigmentation and rate of growth in the colonies of the resistant culture as compared to the sensitive culture

Hebeka & Solotorovsky (1965) consider that the most important cause of the loss of virulence in the resistance cultures is their diminished rate of metabolism whilst the diminution in the rate of growth is of lesser importance This reduction in rate of growth must be assumed to be of considerable importance from the pathogenetic point of view as it is perhaps precisely because of this lower rate of growth that the host organism is able to combat the infection This question must for the present remain open Investigations into the virulence of the resistant cryptococcal cells as compared with that of the original strains in the mouse will play an important part in the elucidation of this problem

SUMMARY

Using Szybalski s gradient plate technique it was found to be relatively easy to induce resistance to nystatin and amphotericin B in five strains of *Cryptococcus neoformans* whilst resistance to trichomycin and polymyxin B was less readily induced

Two out of the three nystatin resistant strains studied were found to show increased cross resistance to amphotericin B whilst cross resistance to nystatin was found in all three of the amphotericin B resistant strains

Changes in colony morphology and dependence on the antimycotic agents were observed in the resistant strains

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THE EFFECT OF MUTAGENS ON THE *m* MARKER OF POLIOVIRUS

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The induction of mutations in poliovirus by mutagen was first studied by *Dulbecco & Vogt* (1958) They examined the effect of proflavine on the mutability of *d* virus to *d* virus (*Vogt et al* 1957) and found that the presence of proflavine during multiplication increased the proportion of *d* mutants in the virus yield *Boeye* (1959) found an increased proportion of *d* survivors when *d* virus was treated with nitrous acid a mutagen much used in phage genetics (*Krieg* 1963) Similar results were obtained with ribonucleic acid from *d* virus and it was also shown that the effect was not due to different rates of inactivation of the *d* and *d* virus by nitrous acid

Nitrous acid has later been found to induce a number of mutations in poliovirus e g from *m* to *m* (*Klein et al* 1966) from large plaques to small plaques (*Carp & Koprowski* 1962 *Ghendon* 1963) from *rct* to *rct* and vice versa and from neurovirulence to nonvirulence and vice versa (*Ghendon* 1963)

Munyon & Salzman (1962) showed that the base analog 5 fluorouracil (5 FU) could be incorporated into the ribonucleic acid of poliovirus Although as much as 36 per cent of the uracils were replaced with 5 FU no change in amino acid composition or plaque morphology could be found *Cooper* (1964) showed however that 5 FU was highly mutagenic if the treated virus was assayed for plating efficiency at supraoptimal temperatures Such 5 FU induced mutants with defective multiplication at high temperatures were used in Coopers work on poliovirus genetics (*Cooper* 1965 *Cooper et al* 1966)

Several other mutagens have been used in phage genetics as reviewed by *Krieg* (1963) e g 2 aminopurine 5 aminoacridine and hydroxylamine

The attenuated vaccine strains of poliovirus type 3 are generally considered less attenuated and genetically more instable than the vac

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cine strains of types 1 and 2 (*e g Vonka et al* 1967) Since it has previously been reported (*Bengtsson* 1966) that eleven attenuated strains of poliovirus type 3 were all *m*+ and since the *m* character may be associated with increased virulence (*Takemoto & Kirschstein* 1964) the effects of some mutagens on the *m* marker of poliovirus type 1 were studied to seek a mutagen able to change the *m* character to *m* Such a mutagen might be used to produce an *m* type 3 strain

The present report shows that several mutagens effect a mutation from *m* to *m* character a mutagen with the reverse effect has not been found

MATERIALS AND METHODS

Cell cultures Primary cynomolgus monkey kidney and HeLa cell cultures were prepared and maintained as described earlier (*Bengtsson* 1966)

Virus Two variants of poliovirus type 1 were used the *m* strain LSc 2ab kindly provided by Dr A Sabin Cincinnati and an *m* variant derived from the *m* strain by isolating a large unhibited plaque under overlay containing 0 01 per cent sodium dextran sulphate Both variants were plaque purified twice Virus stocks were produced in primary monkey kidney cultures

Mutagenic chemicals Nitrous acid was pro analysi from E Merck Darmstadt 2 aminopurine was used as the nitrate salt from Sigma Chemical Company St Louis 5 aminoacridine proflavine HCl and 5 fluorouracil were purchased from K and K Laboratories Inc New York Fresh stock solutions in distilled water were prepared for each experiment 5 aminoacridine was dissolved first in a small amount of 0 1 N HCl All mutagen solutions except nitrous acid were sterilized by boiling for 20 minutes

Virus assay Plaque titration was carried out in plastic petri dishes using HeLa cell cultures The methods and media used have been described earlier (*Bengtsson* 1966) *m* virus was assayed under overlay containing 0 01 per cent sodium dextran sulphate 2 000 (Pharmacia Fine Chemicals Uppsala Sweden)

Counter-current distribution has been described elsewhere (*Bengtsson* 1966) Briefly virus samples were subjected to 19 cycles of transfer in an automatic fractionator (EC Apparatus Company Swarthmore Pa) The phase system used contained 7 per cent (w w) sodium dextran sulphate 500 (Pharmacia Fine Chemicals) 1 2 per cent (w w) polyethylene glycol 6 000 (PEG 6 000 Carbide and Carbon Chemical Co New York) in 0 6 M sodium chloride buffered to pH 7 0 with 0 01 M sodium phosphate buffer

Treatment with nitrous acid One part 4 M $NaNO_2$ and one part 1 M acetate buffer pH 4 5 were mixed with two parts virus suspension After various times at room temperature samples were withdrawn and 1 5 volumes 1 M K_2HPO_4 were added in order to halt the inactivation

Mutagen treatment during virus multiplication Cynomolgus monkey kidney cultures in plastic petri dishes were washed once with PBS and inoculated with 0 1 ml of undiluted virus suspension After 30 minutes adsorption at 37 the cells were washed twice with PBS 5 ml of Eagle s medium (*Eagle* 1959) containing the mutagen was then added and the cultures incubated at 37 for 20 hours The infected cultures were then kept at —60 until assay

RESULTS

Rate of Inactivation and Yield of m and m Virus with Different Mutagens

The inactivating effect of nitrous acid on *m* and *m* virus was first studied to see whether the two variants had different sensitivity Virus samples were incubated with nitrous acid as described in Materials and

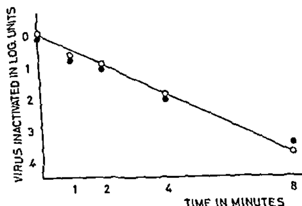


Fig 1

Inactivating effect of nitrous acid on *m* and *m* virus

● – ● *m*⁺ virus ○ – ○ *m* virus

Methods and samples taken at 0 1 2 4 and 8 minutes were assayed for infectivity The results of one experiment are given in Fig 1 There is no difference in the rate of inactivation by nitrous acid between the *m* and *m* variants

The yield of *m* and *m*⁺ virus after multiplication in the presence of mutagens was also examined to see if these variants were inhibited to the same extent The procedure is described in Materials and Methods In the proflavine experiments the cultures were kept in the dark and titrated under red safety light since white light inactivates virus grown in the presence of this dye (*Schaffer* 1962) The effects of four mutagens on the virus yields are shown in Table 1 There is *no significant* difference in the sensitivity of the two virus variants

TABLE 1

Effect of Mutagens on Virus Yield

Virus variant	Yield in per cent of control			
	Proflavine 4 μg/ml	5 aminoacridine 10 μ /ml	2 aminopurine 4 × 10 ³ M	5 fluorouracil 4 × 10 ³ M
m	6 9	0 97	48	3 2
m	7 8	0 84	54	3 5

Detection of m Variants in an m Population

m variants in *m* populations are easily discerned when plated in the presence of dextran sulphate which inhibits the *m* variants In contrast no simple method exists for the identification and isolation of *m* variants in an *m* population It was therefore necessary to devise a method in order to be able to isolate mutagen induced *m* mutants

Acridines Proflavine (3 6 diaminoacridine) has been reported to increase the amount of *d* mutants in the virus yield when present during the multiplication of *d* virus (*Dulbecco & Vogt* 1958) and this marker and the *m* marker are both dependent on the presence of sulphated polysaccharides in the overlay (*Agol & Chumakova* 1962) Proflavine and 5 aminoacridine were therefore studied to see if they could cause a mutation from *m* to *m*

The results of some experiments using different concentration of mutagen are shown in Table 3 It follows that both proflavine and 5 aminoacridine effect a mutation from *m* to *m* and that proflavine is more effective on a molar basis

TABLE 4

Proportion of m Mutants in an m Virus Population after Multiplication in the Presence of 2 Aminopurine or 5 Fluorouracil

Mutagen	*Concentration*	Titre in PFU per 0.1 ml: normal overlay	Titre in PFU per 0.1 ml: overlay containing 0.01% DS	Per cent *m*	mean
2 aminopurine	10^{-3} M	2.3×10^7	4.5×10^4	0.20	
"		1.4×10^7	1×10	0.71	0.43
"		1.8×10^7	7×10^4	0.39	
2 aminopurine	4×10^{-3} M	7×10^8	1.5×10^4	0.21	
"		7×10^8	2×10^4	0.29	0.27
		5×10^8	1.5×10^4	0.30	
5 fluorouracil	10^{-3} M	3.5×10^8	1.4×10^4	0.40	
		6×10^8	7×10^3	0.12	0.23
		4.5×10^8	8×10^3	0.18	
5 fluorouracil	4×10^{-3} M	3.6×10^5	6×10	0.17	
		4×10^5	9×10	0.22	0.26
		5×10	2×10^3	0.40	
Control				0.27	± 0.11

The virus yields in per cent of control were ~ 140 and 50 per cent in the 2 amino purine experiments with 5 fluorouracil they were ~ 35 and 3 per cent

Base analogs 5 fluorouracil (5 FU) is incorporated into poliovirus RNA (*Munyon & Salzman* 1962) and induces poliovirus mutants defective in multiplication at high temperatures (*Cooper* 1964) 2 amino purine is a mutagen much used in phage genetics (*Krieg* 1963) These two analogs were tested at 10^{-3} M and 4×10^{-3} M in multiplication experiments with *m* poliovirus carried out as described above Table 4 shows the results of one experiment No significant increase in the amount of *m* mutants occurs

Attempts to Reveal m to m Mutations

m virus was treated with nitrous acid for 2 minutes as described above or grown in the presence of 2 µg/ml of proflavine or 10 µg/ml of 5 aminoacridine or 10^{-3} M 2 aminopurine or 10^{-3} M 5 fluorouracil The resulting yields were subjected to counter current fractionation before and after multiplication in monkey kidney cultures The fractions were analysed as already described but in no case was the presence of *m* mutants discovered It was concluded that if mutations from *m* to *m* do occur they are not frequent enough to raise the proportion of *m* virus to 0 1 per cent of the total

DISCUSSION

Differences in *m* character probably reflect changes in the protein structure of the virus capsid Such differences have been demonstrated for *m* and *m* variants of encephalomyocarditis virus (*Moscarello & Kaighn* 1964) It is thus to be expected that treatment with mutagens such as nitrous acid which is known to induce changes in the amino acid composition of tobacco mosaic virus (TMV) (*e g Wittman & Wittman Liebold* 1966) should be able to induce mutations from *m* to *m* in poliovirus The present report shows this to be the case and confirms the findings by *Klein et al* (1966) It has also been shown that a mutation from *d* to *d* may be induced by treatment with nitrous acid (*Boeye* 1959) or by the presence of proflavine during virus multiplication (*Dulbecco & Vogt* 1958) Since the *d* and *m* markers are both due to sensitivity to sulphated polysaccharides (*Agol & Chumakova* 1962) the induction of *m* to *m* mutations by nitrous acid and acridines are not surprising

The conclusions from studies on the effect of various mutagens in the rII system of the T4 coliphage are that base analogs and acridines act differently the base analogs inducing transitions *i e* the replacement of a guanine cytosine pair in the phage DNA by an adenine thymine pair or vice versa whereas the acridines induce base pair deletions and/or additions (*Krieg* 1963) There is also very little overlapping between these mutagens *i e* very few mutations are induced by both types of mutagen The findings in this study that the acridines proflavine and 5 aminoacridine induced *m* to *m* mutations whereas the base analogs 2 aminopurine and 5 FU apparently did not are thus in accord with this concept Mutations induced by acridines should however as a rule be reverted by acridines It could thus be expected that mutations from m^{+} to *m* should occur when *m* virus was multiplied in the presence of proflavine and 5 aminoacridine No such mutants were found but the method used for the recovery of possible *m* mutants may have been too coarse since it required the presence of at least 0 1 per cent *m* virus in the *m* population The method used should however be able to reveal a mutation frequency

several times lower than that observed in the reverse system and the failure to find any m^- mutants may also be due to the rarity of such mutations

Nitrous acid is considered to exert its mutagenic action by oxidative deamination of adenine guanine and cytosine In TMV mutants induced by this mutagen the shift in nucleotides from cytosine to uracil was approximately twice as common as a shift from adenine to guanine (*Wittman & Wittman Liebold* 1966) 5 FU induced the conversions uracil cytosine and adenine guanine Since nitrous acid but not 5 FU induced mutations from *m* to *m* , a comparison of the nucleotide shifts produced by these two mutagens in TMV seems to indicate that the change responsible for the mutation *m* to *m* is a conversion of cytosine to uracil It would however then be expected that a conversion of uracil to cytosine should induce the reverse mutation but 5 FU could not be shown to have this effect on m^+ virus The failure to isolate *m* mutants may again be due to an insensitive technique

SUMMARY

Treatment of the *m* virus strain I Sc 2ab with nitrous acid increased the percentage of *m* mutants in the surviving fraction Since an m^+ strain was inactivated at the same rate as the *m* strain the increase is probably the result of mutations The presence of proflavine and 5 aminoacridine during multiplication of the m strain caused an increase of *m* mutants in the progeny while the base analogs 5 fluorouracil and 2 aminopurine did not Methods allowing the recovery of 0 1 per cent *m* virus from a mixture of *m* and *m* virus were used to look for *m* mutants from *m* virus after treatment with nitrous acid or multiplication in the presence of proflavine 5 aminoacridine 5 fluorouracil or 2 aminopurine no such mutants were found

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ATTEMPTS TO MAP THE POLIOVIRUS GENOME BY ANALYSIS OF SELECTED RECOMBINANTS

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Recombination between different strains of poliovirus was first demonstrated by *Hirst* (1962) by the use of crosses between horse serum resistant and bovine serum resistant strains recombinants were able to grow in the presence of both inhibitory sera The results were confirmed by *Ledinko* (1963) who used strains resistant to guanidine and horse serum The recombinants were found to be approximately 0 4 per cent of the total progeny This recombination rate is low compared to that of DNA phages (*e g Stent* 1963) and may be explained by the small size of the viral RNA and by the finding that poliovirus synthesis takes place in cytoplasmic vesicles (*Becker et al* 1963) which limits the exchange of genetic material Although *Cooper* (1967) has reported up to 1 per cent recombination in crosses between poliovirus *ts* mutants which do not replicate at high temperatures it would not seem practical to use differences in recombination rates between various poliovirus strains for mapping the poliovirus genome

Cooper et al (1966) have used a different method for genome mapping Using 5 fluorouracil as a mutagen several *ts* mutants of poliovirus have been obtained (*Cooper* 1964) When grown above the temperature allowing normal multiplication these mutants differ in their ability to induce changes in cell metabolism such as prevention of cellular incorporation of labeled thymidine into DNA prevention of P^{32} incorporation into cell RNA synthesis of viral RNA and appearance of viral antigen The *ts* mutants could then be divided into four groups depending on the functional defect Further work along these lines has resulted in a tentative physiological map of the *ts* mutants (*Cooper* 1967)

This paper reports the recombination between guanidine resistant variants and horse serum resistant variants where the variants also

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differ with respect to another genetic marker such as the m marker (Nomura & Takemori 1960) The fraction of recombinants positive for the additional marker indicates the distance between the marker sites in the viral genome As it was not possible to use variants with identical mutations the results must be evaluated with the reservation that the marker sites may differ in the two variants i e the same character may be the result of mutations at different sites in the viral genome

MATERIALS AND METHODS

Virus All virus variants used were derived from the attenuated poliovirus type 1 strain LSc 2ab kindly provided by Dr A Sabin Cincinnati Ohio It was plaque purified twice in this laboratory This strain is sensitive to guanidine hydrochloride (*gu*) (*Rightsel et al* 1961) and inhibitory horse serum (*ho*) (*Takemoto & Habel* 1959) inhibited by dextran sulphate (*m*) (*Takemoto & Liebhaber* 1962) does not multiply normally at temperatures such as 38 or 39 C (*rct*-) (*Lwoff* 1959) and is inactivated at 50 C (*t*-) (*Youngner* 1957)

Cell cultures and virus assay Primary cynomolgus monkey kidney cultures grown as described elsewhere (*Bengtsson* 1966) were used for production of virus stocks and for recombination experiments

Virus infectivity was determined by plaque titration in HeLa cells as has been described earlier (*Bengtsson* 1966)

Chemicals Guanidine hydrochloride was reinst 100 per cent from Dr Theodor Schuchardt Munich Germany Dextran sulphate (Dextran sulphate 2000) was a generous gift from Pharmacia Fine Chemicals Inc Uppsala Sweden

Horse serum Horse serum samples furnished by the National Bacteriological Laboratory Stockholm Sweden were screened for inhibitory activity at a concentration of 10 per cent in agar overlay One serum designated 1622 was found to be markedly inhibitory A large batch of this was Seitz filtered and kept frozen a —20 for further use It was used unheated througout the investigation

Production of Virus Variants

gu A *gu* variant was obtained by growing the LSc 2 ab strain in monkey kidney cells containing increasing amounts of guanidine until the plaque titre in HeLa cell cultures with 5 × 10-4 M guanidine in the overlay was the same as that in control cultures Attempts to isolate one step mutants by picking plaques under 5 × 10-4 M guanidine failed Such plaques were not resistant to guanidine

ho A *ho* virus variant resistant to serum 1622 was produced by repeated passage in monkey kidney cultures with 10 per cent serum 1622 until the plaque titre in HeLa cells under overlay with 6 per cent serum 1622 was the same as that in controls One step mutants could not be isolated

m *m* variants were isolated separately from the *gu* and *ho* strains by plating them under overlay containing 0 01 per cent dextran sulphate Plaques that were not inhibited by the polyanion were passed in monkey kidney cultures The variants were designated *gu ho* m_1 and *gu ho m*

rct+ *rct 39* virus capable of growing at 39 C was produced from the *gu* and *ho* variants by growing them at increasing temperatures in monkey kidney cultures until the plaque titre at 39 C was the same as that at 37° C Some experiments used *rct 38* variants obtained in a similar manner The variants were numbered in the same manner as the *m* variants

t *t* variants were obtained by heating *gu* and *ho* virus samples to 50 C for 30 minutes before growth in monkey kidney cultures This procedure was repeated once When the plaque titres of the virus yields before and after heating for 30 minutes at 50 C were compared the *ho* variant was found to be decreased approximately 1 log whereas the *gu* variant was still very sensitive After a further five passages in monkey kidney cells alternating with heat inactivation the difference between titre before and after inactivation was still about 2 log for the *gu* strain but the difference for the original *gu* strain was > 4 log and the heat

resistance of the variant was considered sufficient for use Both variants were numbered as already described above

All virus variants obtained were then plaque passaged twice in HeLa cells and virus stocks produced in monkey kidney cultures were frozen and kept at —60 C until use

Marker Tests

m character A recombinant was regarded as *m* if the difference between the plaque titre with and without 0 01 per cent dextran sulphate in the overlay was not more than 1 0 log

rct character was measured by comparing plaque titres at 37 C and 39 C and in some cases at 38 C The criteria for an *rct*+ character at the temperatures used was a plaque titre not more than 1 0 log lower at the higher temperature than at 37 C

t character Virus samples were incubated at 50 C for 30 minutes They were then transferred to an ice bath and assayed for infectivity together with unheated controls The criteria for *t* character was a decrease in titre not more than 1 5 log after heating when the *ho* t_s variant was used and less than 2 5 log when the *gu* t_i variant was used

RESULTS

Characterization of Virus Variants Used

All virus variants used in the recombination experiments were analyzed for their marker characteristics They were also tested for plating efficiency under overlay containing 5×10^{-4} M guanidine 6 per cent serum 1622 and the same amounts of guanidine plus serum

The results are shown in Table 1 The *rct 39* strains were also *m* and to a certain extent *t* Also the inhibitory effect of horse serum on serum sensitive strains is slightly greater than that of guanidine on guanidine sensitive strains at the concentrations used The titrations under overlay with both inhibitors showed the presence of background mutants resistant both to guanidine and to horse serum The frequency of such mutants was however always lower than the recombination frequencies obtained in the crosses between variants as will be shown later

Recombination experiments were performed in roller tubes containing between 10 and 2 × 10 monkey kidney cells Tubes were inspected under a microscope and the cell side marked They were then washed once with phosphate buffered saline (PBS) and inoculated with 0 2 ml of the virus mixture to be used which contained approximately equal amounts of the two viruses The multiplicity for both virus variants was at least 50 plaqueforming units/cell The tubes were placed with the cell side downwards and allowed to adsorb virus for 30 minutes at 37 They were then washed twice with PBS and supplied with 1 ml of Hank s salt solution containing 0 5 per cent lactalbumin hydrolysate After incubation in rollers for 17 hours the tubes were frozen at —60 until assay

Isolation of Recombinants

The yields from recombination experiments were analysed by plaque assay under 5×10 M guanidine 6 per cent inhibitory horse serum

TABLE 1

Characteristics of the Ts ab Variants used in Recombination Experiments

Variant	Titre in log PFU/0 1 ml under ordinary overlay	Titre decrease in logs when titrated at 33 C	Titre decrease in logs when titrated at 38 C	with 0 01 per cent DS in overlay	after heating at 50 C for 30 min	with 5×10⁻⁴ M GHCl† in overlay	with 6 per cent serum 16?2 in overlay	with 5×10⁻⁴ M GHCl and 6 per cent 16?? in overlay	Background mutants per cent
gu ho	7 1	>4 9	>?0	? ?	4 1	+0 2	>4 1	4 4	0 004
gu ho	7 2	4 3	>?0	3 0	4 4	4 ?	0 0	4 7	0 002
gu ho m₁	7 ?	>5 ?	ND§	+0 3	4 0	+0 2	>4 2	>4 2	<0 006
gu ho m₂	7 1	>5 3	ND	+0 3	4 1	4 0	0 1	4 5	0 003
gu ho t₁	7 ?	>4 7	ND	? 6	1 7	+0 1	>4 ?	>4 2	<0 006
gu ho t₂	6 7	>4 C	ND	3 0	0 9	4 1	0 2	4 5	0 003
gu ho ret 38₁	7 1	>3 7	0 3	ND	ND	+0 1	>4 1	>4 1	<0 009
gu ho ret 38₂	7 0	>4 0	0 1	ND	ND	4 1	0 1	4 -	0 003
gu ho ret 39₁	7 1	0 ?	ND	+0 1	1 9	+0 1	>4 1	>4 1	<0 009
gu ho ret 39	8 0	0 4	ND	0 0	1 7	3 4	0 0	4 0	0 01

DS — dextran sulphate

† GHCl = guanidine hydrochloride

§ ND = not done

resistance of the variant was considered sufficient for use. Both variants were numbered as already described above.

All virus variants obtained were then plaque passaged twice in HeLa cells and virus stocks produced in monkey kidney cultures were frozen and kept at −60 C until use.

Marker Tests

m character. A recombinant was regarded as *m* if the difference between the plaque titre with and without 0.01 per cent dextran sulphate in the overlay was not more than 1.0 log.

rct character was measured by comparing plaque titres at 37 C and 39 C and in some cases at 38 C. The criteria for an *rct*⁺ character at the temperatures used was a plaque titre not more than 1.0 log lower at the higher temperature than at 37 C.

t character. Virus samples were incubated at 50 C for 30 minutes. They were then transferred to an ice bath and assayed for infectivity together with unheated controls. The criteria for *t* character was a decrease in titre not more than 1.5 log after heating when the *ho* t_g variant was used and less than 2.5 log when the *gu* t_r variant was used.

RESULTS

Characterization of Virus Variants Used

All virus variants used in the recombination experiments were analyzed for their marker characteristics. They were also tested for plating efficiency under overlay containing 5×10^{-4} M guanidine, 6 per cent serum 1622 and the same amounts of guanidine plus serum.

The results are shown in Table 1. The *rct 39* strains were also *m* and to a certain extent *t*. Also the inhibitory effect of horse serum on serum sensitive strains is slightly greater than that of guanidine on guanidine sensitive strains at the concentrations used. The titrations under overlay with both inhibitors showed the presence of background mutants resistant both to guanidine and to horse serum. The frequency of such mutants was however always lower than the recombination frequencies obtained in the crosses between variants as will be shown later.

Recombination experiments were performed in roller tubes containing between 10 and 2×10 monkey kidney cells. Tubes were inspected under a microscope and the cell side marked. They were then washed once with phosphate buffered saline (PBS) and inoculated with 0.2 ml of the virus mixture to be used which contained approximately equal amounts of the two viruses. The multiplicity for both virus variants was at least 50 plaque forming units/cell. The tubes were placed with the cell side downwards and allowed to adsorb virus for 30 minutes at 37. They were then washed twice with PBS and supplied with 1 ml of Hanks salt solution containing 0.5 per cent lactalbumin hydrolysate. After incubation in rollers for 17 hours the tubes were frozen at −60 until assay.

Isolation of Recombinants

The yields from recombination experiments were analysed by plaque assay under 5×10 M guanidine 6 per cent inhibitory horse serum

TABLE 2

R lt f R o b at on Experi t th Variants

	Number of tube cultures infected	Mean titre in log PFU/0 1 ml with			Per cent recombinants	Number of recombinants		Per cent
		5×10 4 M CHCl	6 per cent serum 1622	5×10 4 M GHCl + 6 per cent serum 1622		*m*	*m*	*m*
	10	5 4	6 4	3 9	0 25	34	0	100
		6 4	6 4	4 3	0 40	80	0	100
		6 2	6 5	3 7	0 10	4	19	17
	2	6 4	6 4	3 8	0 14	7	67	9

TABLE 3

Results of Recombination Experiments with t Variants

	Number of tube cultures infected	Mean titre in log PFU/0 1 ml with			Per cent recombinant	Number of recombinants		Per cent
		5×10 4 M CHCl	6 per cent serum 1622	5×10 4 M GHCl + 6 per cent serum 1622		*t*	*t*	*t*
gu ho t × *gu ho* t_s	12	6 4	6 1	4 0	0 28	50	12	81
gu ho t × *gu ho* t_s	8	5 8	5 8	3 6	0 3	40	5	93
gu ho t_r × *gu ho t*	20	5 7	6 6	3 6	0 08	9	51	14
gu ho t_r × *gu ho t*	16	6 6	5 6	3 3	0 0	2	37	

1622 and guanidine together with serum 1622 Recombinant plaques characterized by their larger size under overlay containing both guanidine and horse serum were picked transferred to tubes containing 0 25 ml of PBS and frozen at —60 Material from the tube was then again plaque titrated under guanidine and horse serum and one new plaque selected in the same manner Material from this was then subjected to marker tests

The genetic stability of the isolated recombinants was also tested by plaquing one recombinant under ordinary overlay and examining 25 progeny plaques In no case was the difference between the plaque titre under ordinary overlay and inhibitory overlay > 0 3 log the mean titres differed by 0 05 log units The recombinants were thus considered to be genetically stable

Experiments with m Variants

Recombination experiments were carried out by infecting monkey kidney tube cultures with a mixture of *gu ho m* and *gu ho m* virus as well as *gu ho m* and *gu ho m* virus at high multiplicities as described above The yields were then plaque titrated in order to determine the titre of *gu ho* and recombinant *gu ho* virus Recombinant plaques were picked plaqued and further analyzed as already described to determine the proportion of recombinants resistent to dextran sulphate i e *m* in the two kinds of recombinations The results of two such experiments of each kind are shown in Table 2

It is evident from this Table that the frequency of *m* recombinants differs in the two types of recombination experiments In the cross between *gu ho m* and *gu ho m* virus all recombinants were *m* So far no *m* recombinant has been found in this type of cross out of more than 200 tested In the other cross between *gu ho m* and *gu ho m* virus approximately 11 per cent of the recombinants were *m* (11 out of a total of 97) The recombination rates were found to be higher in the cross *gu ho m* × *gu ho m* than in the cross *gu ho m* × *gu ho m* A comparison with Table 1 shows than their frequency is 25 times higher or more than the background mutations of the virus variants used

In experiments of this kind where recombinants are tested for a third genetic marker from one parent the fraction possessing this third character is directly related to the marker distances in the viral genome provided that the frequency of double recombination is negligible In the cross between *gu ho m* and *gu ho m* virus ~ 11 per cent of the recombinants were *m* which means that only about 11 per cent of all breaks leading to the appearance of *gu ho* recombinants took place between the *m* marker and the *ho* marker In the other cross with *gu ho m* × *gu ho m* virus where all recombinants were *m* all breaks occurred between the *gu* and the *m* marker The results

TABLE 4

Results of Recombination Experiment with gu ho m_1 t and gu ho m t_2 Variants

Number of tube cultures	Mean titre in log PFU/0.1 ml with: 5×10⁻⁴ M CHCl	6 per cent serum 1692	5×10⁻⁴ M CHCl + 6 per cent serum 1692	Per cent recombinants	Number of recombinants: *m t*	*m t*	*m t*	*m t*	Per cent *m*	Per cent *t*
9	[illegible]	6.7	4.1	0.11	17	0	25	4	39	61

TABLE 5

Results of Recombination Experiments with ret 39 Variants

Cross		Number of tube cultures infected	Mean titre in log PFU/0.1 ml with: 5×10⁻⁴ M CHCl	6 per cent serum 1692	5×10⁻⁴ M CHCl + 6 per cent serum 1692	Per cent recombinant	Number of recombinants: *ret 39*	*ret 39*	Per cent *ret 39*
gu ho ret 39	× *gu ho ret 39*	[illegible]	6.4	6.7	1.9	0.03	18	4	92
gu ho ret 39	× *gu ho ret 39*	19	6.4	6.9	3.8	0.06	24	4	86
gu ho ret 39	× *gu ho ret 39*	8	6.3	6.6	3.2	0.027	24	3	[illegible]
gu ho ret 39	× *gu ho ret 39*	16	6.5	6.6	3.0	0.024	16	4	[illegible]

results show that the first alternative m_1 between l_4 and *ho* is probably correct

Experiments with rct Variants

Recombinants from the crosses *gu ho rct 39* $^-$ × *gu ho rct 39*$_1$$^\circ$ and *gu ho rct 39*$_1$ × *gu ho*$^+$*rct 39*$^{\circ -}$ were tested for their *rct 39* character as described in Materials and Methods The results are shown in Table 5 The recombination rates were much lower than those in the experiments already described They were however at least 9 (*gu ho rct 39* × *gu ho rct 39*$_2$ $^\circ$) or > 4 (*gu ho rct 39*$_1$ $^+$ × *gu ho*$^+$*rct 39*) times more frequent than the background mutants shown in Table 1 The proportion of *rct 39* $^+$ recombinants is very high in both types of cross *This would be the case if the rct 39 character were due to several* markers on the virus genome Complementation could then occur and only recombinants that lack one or more markers would be *rct* If this is correct an *rct 38* virus may well possess some of these markers and recombination between such strains could produce recombinants that were *rct 39* $^+$ To test this hypothesis a recombination experiment was carried out with *gu ho rct 38*$_1$$^\circ$ × *gu ho rct 38* virus A cross between *gu ho* and *gu ho* served as controls The recombinants were tested for *rct 39*$^\circ$ character and the results are given in Table 6 Approximately 16 per cent of the recombinants in the cross between *gu ho rct 38*$_1$$^\circ$ and *gu ho rct 38*$_2$$^\circ$ are *rct 39* In the experiment *gu ho* × *gu ho* no *rct 39* recombinant was found

DISCUSSION

In this work the *gu* and *ho* variants from which the other variants were derived had to be multistep mutants isolated after several cycles of growth in the presence of inhibitors The *gu* and *ho* characters are therefore probably the result of mutations at several sites They are *however identical for all variants and since the aim was to determine* the relative locations of the markers no other method was available The recombination frequencies in this study varied the highest rates were observed in crosses with *m* mutants and the lowest in those with *rct 39* mutants The recombination frequencies were however always *high enough to differ significantly from the frequencies of* double resistant background mutants Only in one cross *gu ho m* × *gu ho m* the frequency (0 40 per cent) was as high as that reported by *Ledinko* (1963) for recombination with *gu* and *ho* virus and by *Hirst* (1962) for recombination with *ho* and *ho* Since all recombinations in this study were selected for *gu* would have been expected to be more or less other marker characters present in the variant nation rates in the experiments with *rct 39* prising One possible explanation could

TABLE 6

ret 39 Character of Recombinants from the Cross gu ho ret 38₁ × gu ho ret 39₂

ro s	Number of tube cultures infected	Mean titre in log PFU/0 1 ml with 5×10⁻⁴ M CHCl	6 per cent serum 1622	5×10⁻⁴ M CHCl + 6 per cent serum 1622	Per cent recombinants	Number of recombinants ret 39	ret 39	Per cent ret 39
gu ho ret 39₁ × gu ho ret 38₂	6	6 1	6 6	3 9	0 17	6	31	1[illegible]
gu ho × gu ho	6	6 2	6 7	4 1	0 17	0	[illegible]3	0

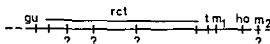


Fig 2
Tentative map of the poliovirus genome

more stable in virus able to multiply at higher temperatures and is thus less liable to give recombinants *Cooper* who has reported the highest rates of recombination with poliovirus so far up to 1 per cent worked with *ts* mutants (*Cooper* 1967) which may be less stable than ordinary virus

If a character had only one single marker site in the viral genome addition of the percentages of recombinants positive in the two types of experiments should be ~ 100 per cent This was the case in the experiments with the two *t* mutants The *t*⁺ variants are however multistep mutants probably with several marker sites although the results indicate that these sites are located very close together and may be identical With *m* virus all recombinants in the cross *gu ho m* × *gu ho* m_2 were *m* This would be the result if the *ho* marker were located between the *gu* and the m_2 marker If the *m* marker sites were identical presence of *m* recombinants in the cross *gu ho* m_1 × *gu ho m* would then have to be the result of double recombinations The poliovirus genome is however considered to have no more than 14 genes (*Summers et al* 1965) and the frequency of *m* recombinants was as high as ~ 11 per cent The existence of two different marker sites one on each side of the *ho* marker was therefore considered a more likely explanation On the other hand the results could also be explained by a linkage between the *m* and *ho* markers Crosses with *rct 39* variants showed frequencies of *rct 39* recombinants above 90 per cent in both types of experiment This was interpreted to be due to the existence of several marker sites which agrees with the results of *Cooper et al* (1966) and *Cooper* (1967) who found five *ts* mutant groups The finding that approximately 16 per cent of the recombinants in an *rct 38* cross were *rct 39* also agrees with this hypothesis

Taken together the findings in this study suggest the tentative genetic map shown in Fig 2 This is in accordance with the fact that guanidine prevents the appearance of one early gene product the viral RNA polymerase for sensitive virus (*Baltimore et al* 1963) The *m* marker which is probably identical with the *d* marker (*Agol & Chumakova* 1961) has been shown to be due to differences in affinity to polyions such as dextran sulphate (*Bengtsson & Philipson* 1963 *Bengtsson* 1965) and ion exchangers (*Bengtsson et al* 1964) The reason for this difference is probably due to differences in one of the four polypeptides of the viral coat found by *Mai et al* (1963) and *Summers et al* (1965) a difference in the amino acid composition of *m* and *m*

mutants of encephalomyocarditis virus has been demonstrated by *Moscarello & Kaighn* (1964) The basis for the different rates of inactivation of *t* and *t* virus (*Youngner* 1957) at 50 C has not been much studied but *Dimmock* (1967) recently stated that inactivation at high temperatures (55 C) was due to damage to the coat protein and that heated poliovirus does not bind antibody Inhibitory horse serum has been found to cause a decrease in viral adsorption rate (*Takemoto & Habel* 1959) and *Pagano* (1965) found that sensitive virus combines with this inhibitor Both the *t* and *ho* markers can therefore be based on different compositions of the coat protein The arrangement of the *m* *t* and *ho* markers on the tentative map thus agrees with the theory that they are all due to differences in the composition of the protein coat

SUMMARY

The poliovirus type 1 strain LSC 2ab was used to produce *gu* and *ho* variants with or without *m* *rct 39* + or *t* markers These were used to make *gu ho* recombinants which were isolated in the presence of guanidine and inhibitory horse serum The frequency of recombinants varied between 0 024 and 0 40 per cent but was always higher than the frequency of background double mutants The recombinants were studied for *m* , *rct 39* + or *t* character with marker tests

The results obtained were explained as follows

1) The *t* character is due to mutations at several marker sites close to each other and located between the *gu* and *ho* markers close to the latter
2) The *m* character is due to different mutations in the two *m* variants used with one marker site on each side of the *ho* marker One marker site was located between the *t* and *ho* markers
3) The *rct 39* character is due to mutational changes at several marker sites between the *gu* and *ho* markers

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HUMORAL ANTIBODIES IN CANINE RENAL TRANSPLANTATION

By

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Many competent investigators hold the view that allograft rejection is primarily mediated by cells rather than by circulating antibodies (*Billingham & Brent* 1956 *Russell & Monaco* 1965) This opinion is partly based on the difficulties in detecting serum antibodies against the grafted tissue at the time of rejection (*Govaerts* 1960 1964) Such antibodies were demonstrable only after hyperimmunization (*Terasaki* 1959 *Stetson & Jensen* 1960 *Kapitchnikow et al* 1962 *Altman & Simonsen* 1964) The efforts aimed at a demonstration of circulating antibodies have however continued stimulated by studies of the rapid second set rejection where little evidence of cellular infiltration is seen

Recently certain findings have pointed to the existence and probable importance of circulating antibodies in graft rejection Thus transplantation immunity has been passively transferred by allogeneic immune serum (*Altman* 1963 *Najarian & Perper* 1967) Furthermore *Kretschmer & Pere Tamayo* (1962) and *Najarian & Feldman* (1962) have reported the transfer of sensitivity to skin homografts by means of large numbers of isologous sensitized lymphoid cells enclosed in Millipore chambers impermeable to cells Other investigators (*Fujimoto et al* 1966 *Wilson et al* 1966) have however been unable to provide evidence that humoral antibodies can cause homograft destruction in the absence of sensitized recipient cells

Leucocyte agglutinins haemagglutinins cytotoxic antibodies and antibodies measured by their antiglobulin consumption have been detected after skin allotransplantation (*Terasaki* 1959 *Altman & Simonsen* 1964 *Colombani et al* 1964 *Pu a et al* 1964 and *Kuhns et al*

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1964). In contrast it has been difficult to demonstrate circulating antibodies against donor cells after allogeneic kidney transplantation. Recently, however, evidence has accumulated which suggests that humoral antibodies may also play a significant role in kidney allotransplantation (*Terasaki et al.* 1961, *Altman* 1963, *Milgrom et al.* 1966, *Porter* 1967, *Williams et al.* 1967, *Rapaport et al.* 1967, *Iwasaki et al.* 1967). Of particular interest are the descriptions of hyperacute kidney rejection in patients who have pre-existing humoral antibodies against donor cell antigens (*Kissmeyer-Nielsen et al.* 1966, *Williams et al.* 1967).

The present paper describes the demonstration of humoral antibodies appearing as a result of renal allotransplantation in dogs. In particular the kinetics of the production and the specificity of such antibodies detectable by four different serological methods have been studied.

MATERIALS AND METHODS

Transplantation Technique

Eight allogeneic renal transplants and two second set skin transplants were performed on mongrel dogs weighing 10 to 15 kgs. As a rule, donor and recipient belonged to the same litter. The recipients received no immunosuppressive therapy.

Anaesthesia was induced by thiopenthal sodium. A pulmomat or respirator was used. Serum samples were drawn from the donors and recipients before the transplantation and from the recipients at certain time intervals after transplantation. The kidney grafts were anastomosed end to end to the recipient vessels and the ureter was anastomosed to the bladder. In three cases urography was performed to check the function of the graft. In three cases the second donor kidney was extirpated and used for cell cultures and in five cases donor bone marrow was removed and the cells cultivated. When required for serological testing, blood was drawn from the donor and the lymphocytes were isolated by the gelatin method (*Coulson & Chalmers* 1964).

Test for Cytotoxic Antibodies

Threefold serial dilutions of the test sera, heated at 56° C for 30 minutes, were made in medium Parker 199 containing 0.000 M Mg^{++} and 0.00015 M Ca^{++}. To 0.05 ml of each serum dilution was added 0.05 ml cell suspension (about 100 000 cells) and then 0.05 ml guinea pig serum diluted 1:2 in Parker 199. Controls included donor cells incubated with Parker 199 and guinea pig complement, donor cells incubated with donor serum and complement, donor cells incubated with recipient serum and heated complement and finally recipient cells with autologous serum and complement. The mixtures were incubated for 30 minutes at 37° C and 0.05 ml of a 1:400 dilution of trypan blue in physiological saline was added. After 15 minutes ≥ 200 cells from each tube were examined and the percentage of coloured cells (cytotoxicity) was calculated.

The procedure was rather similar when Cr^{51} labelled lymphocytes were used as target cells (*Sanderson* 1964, *Wigzell* 1965). The cells were incubated with sodium chromate (20 μC/10^{6} cells) for 2 hours at 37° C and washed three times with Parker 199. Before the last washing the cells were left at 4° C for 20 minutes. 0.05 ml cell suspension was mixed with twofold dilutions of the test sera (0.05 ml) and rabbit serum diluted 1:2 in Mg^{++} and Ca^{++}-containing Parker 199 (0.05 ml) was added. The tubes were incubated at 37° C for 60 minutes, the cells pelleted and the supernatant assayed for release of Cr^{51} in a Nuclear Chicago Bi-pan FM scintillation detector.

Anti-Dog Globulin Serum

Rabbits were injected intramuscularly with 60 mg of ammonium sulphate precipitated dog gamma globulin in Freund's complete adjuvant; the animals were

rested for one month and boostered twice subcutaneously with 2 mg of dog gamma globulin at weekly intervals The rabbits were then rested for two months boostered twice subcutaneously and finally bled two weeks after the last injection

Amboceptor

Dog anti sheep erythrocyte serum was produced by injecting a dog intramuscularly with 1 to 3 ml of a 20 per cent suspension of washed sheep erythrocytes at weekly intervals for 6 weeks The final bleeding was done 10 days after the last injection The haemagglutination titre of this serum when tested against a 0 2 per cent suspension of sheep erythrocytes was 1280

Coated Indicator Cells

Washed sheep erythrocytes were coated with amboceptor by incubating aliquots of the dog anti sheep erythrocyte serum at a dilution of 1 1000 and a 2 per cent cell suspension for 1 hour at room temperature The cells were then washed twice with physiological saline resuspended to the original concentration and incubated with an equal volume of diluted (1 180) anti dog γ globulin serum for 1 hour at room temperature To find the most suitable dilutions of amboceptor and anti dog globulin serum to employ in the mixed haemadsorption tests red cells were coated with varying dilutions from each serum and these indicator cells were then used to trace dog antibodies produced against allogeneic dog kidney cells by hyperimmunization Indicator cells which gave a positive reaction at a high dilution of the immune serum but no or only a trace of haemadsorption with normal undiluted dog serum were selected for the haemadsorption tests

Mixed Haemadsorption Tests

When kidney cells and occasionally bone marrow cells were used as target cells mixed haemadsorption (*Espmark* & *Fagraeus* 1962 *Fagraeus et al* 1965) was performed as follows Monolayer cell cultures in milk dilution bottles or in plastic petri dishes were washed with Eagle s basal medium and overlaid with a 3 mm deep layer of 0 5 per cent Difco agar in Eagle s medium with 5 per cent calf serum Sterilized filter paper discs 5 mm in diameter were allowed to absorb 0 02 ml of the inactivated test serum and placed 20 mm apart on the agar surface The flasks and the petri dishes were incubated at 37 C (the dishes in humidified 6 per cent CO_2 in air) for 24 hours physiological saline was added and the agar layer poured off A suitable volume of a 2 per cent suspension of indicator cells was added and left on for 2 hours at room temperature The supernatant was then cautiously removed and the diameters of the adsorption zones were measured Occasionally the flasks or petri dishes were dried and stained with benzidine prior to measuring the zones

A modification of the above procedure was applied to blood lymphocytes and often also to bone marrow cells Since these cells attach poorly to glass or plastic they were fused with HeLa cells prior to cultivation The two cell types were mixed in a ratio of 1 1 (HeLa & bone marrow) or 1 2 (HeLa & lymphocytes) and 200 to 800 haemagglutinating units (HAU) of UV irradiated Sendai virus was added per 10^6 cells The cell suspension was shaken incubated for 30 minutes at 4 C and seeded on petri dishes which were placed in a 37 C incubator containing humidified 6 per cent CO_2 in air After two days incubation the cell ltures were washed with Eagle s basal medium and from then on the pro edure d b d b e was followed

Haemagglutination Tests for Heterophile Antibod e

Heat inactivated te t sera w re diluted ser all in gl al line buffered with 0 0 M phosphate (PBS) to pH To 0 4 ml f un d lution was added 0 ml of a 0 5 per cent erythroc te suspens on and tr t bes were examined for haemagglutination after 2 hours incubation t r om temperature

Cells

a) kidney cells the kidney c psule was remo ed the tissue was ut in small pieces washed trypsinized and the cells were cultivated in Eagle b al medium with 15 per cent calf serum.

b) *Bone marrow cells* a piece of bone marrow was dispersed with syringe and needle filtered washed three times and cultivated in Eagle s basal medium with 15 per cent foetal calf serum

c) *HeLa cells fused with bone marrow cells or blood lymphocytes* Eagle s basal medium supplemented with 10 per cent foetal calf serum was used

d) *Blood lymphocytes* three volumes fresh blood was mixed with one volume 3 per cent gelatine solution (Crude L 936/B AB Analyskemiska Stockholm) and incubated at 37 C for ½ hour The top phase was collected the cells pelleted and washed twice in physiological saline To the pelleted cells was added one ml of distilled water to lyse the red cells and isotonicity was restored after 10 to 15 seconds by the addition of one ml 1 8 per cent physiological saline The cells were washed once more and counted.

e) Erythrocytes were washed three times and stored in Claus-Jensen buffer prior to use

Sendai Virus

Sendai virus was propagated in the allantoic cavity of 10 to 11 days old embryonad chicken eggs The eggs were inoculated with 0 5 to 1 HAU of virus incubated for 2 days at 36 C transferred to 4 C overnight and the allantoic fluid was collected The fluid was filtered though paper dialysed against TBS overnight and filtered in two steps through Millipore filters 0 80 μ and 0 45 μ The sterile virus preparation was centrifuged at 30000 g for 30 minutes in a Spinco ultracentrifuge and the pelleted virus particles were resuspended in Parker 199 devoid of glucose to 1/10 of the original volume The virus preparation was finally distributed in 2 ml ampoules and frozen at —20 C. The haemagglutination titres of stock virus preparations when tested against 0 2 per cent human (O+) erythrocytes ranged between 4000 and 5000 HAU/ml

Prior to use in cell fusion experiments Sendai virus was UV irradiated for 3 minutes in a constantly agitated Petri dish placed 70 cm from a 15 watt Sylvania germicidal lamp

RESULTS

Cytotoxic Antibodies Measured in the Dye Test or Using Cr⁵¹ Labelled Target Cells

Recipient sera were examined in the dye test using donor bone marrow cells kidney cells or blood lymphocytes as target cells Six tested recipients all demonstrated a weak transient antibody response as early as 5 to 7 days after transplantation Fig 1 shows the average cytotoxic activity of five recipient sera against donor bone marrow cells The decline in antibody titres during the second week after transplantation coincided with the rejection of the grafted kidney Very low cytotoxic activity was demonstrable up to two months after transplantation The antibody titres were similar when donor bone marrow and kidney cells were used but the peak titres were reached somewhat later when kidney cells were the target cells Absorption of recipient serum with donor bone marrow cells completely abolished the cytotoxic antibody activity Reduction of recipient sera with 0 2 M 2 mercaptoethanol decreased but did not abolish the cytotoxic activity

The preliminary results obtained by using Cr⁵¹ labelled donor lymphocytes are nearly identical to those from the dye test Rabbit serum as the source of complement has been found superior to guinea pig serum

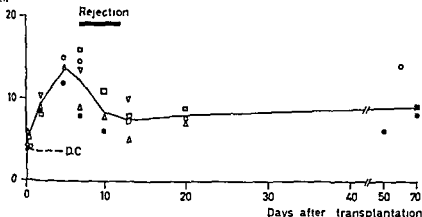


Fig 1

Cytotoxic antibody activity following renal transplantation measured in the dye test using donor bone marrow cells as target cells The curve represents the average cytotox effect of serum samples from five recipients A low percentage (3–5) of cells were damaged during their preparations as seen in the DC control (donor serum and syngeneic bone marrow cells)

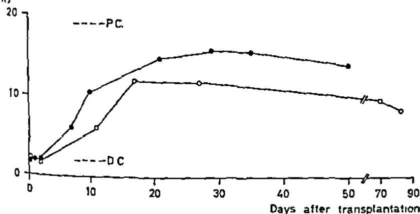


Fig 2

Kinetics of the antibody response of two recipients following renal transplantation measured by the mixed haemadsorption t t T rget cells were donor kidney cells Donor serum and syngeneic kidney ll D ( ) g e weak nonspecific haemad sorption zones not exceeding 2–3 mm ham ter PC = dog anti dog kidney immune serum a d l n r ki lney cells

Antibodies Detectable by the Mixed Haemadsorption Technique

Kidney bone marrow cells and blood lymphocytes have been em ployed in this test The lymph cytes and occasionally the bone marrow cells were fused with HeLa cell by UV irradiated Sendai virus prior to testing When donor kidney l were the target cells humoral anti

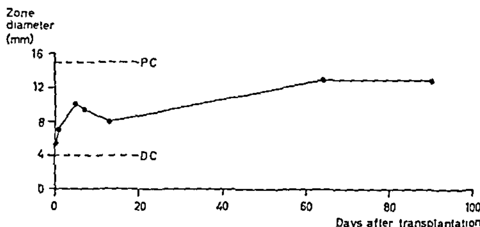


Fig 3

Kinetics of the antibody response following renal transplantation measured by the mixed haemadsorption test Target cells were donor bone marrow cells fused with HeLa cells by Sendai virus Donor serum and syngeneic bone marrow cells fused with HeLa cells (D C.) gave weak nonspecific haemadsorption zones not exceeding 4 mm s diameter P C. = donor anti dog kidney serum and donor bone marrow cells fused with HeLa cells

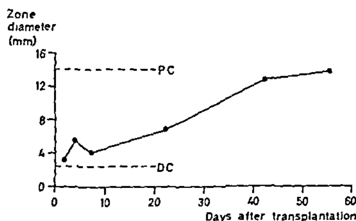


Fig 4

Kinetics of the antibody response following renal transplantation measured by the mixed haemadsorption test Target cells were donor bone marrow cells D C = donor serum and syngeneic bone marrow cells P C = dog anti dog kidney serum and donor bone marrow cells

bodies were not detectable in significant amounts until 7 to 10 days after transplantation (Fig. 2) At this time the circulation of the kidney transplant was already impaired and rejection had begun Subsequent to rejection the antibody titres increased rapidly for about two weeks and then decreased slowly for several weeks Thus an early (4 to 6 days after transplantation) transient antibody response similar to that observed in the cytotoxic antibody assays was not observed Absorption of recipient sera with donor kidney cells caused a significant decrease

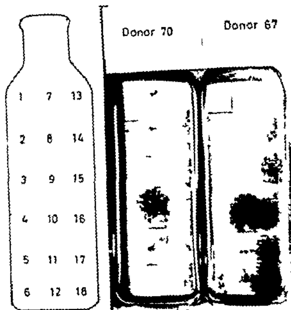


Fig 5

The use of the mixed haemadsorption test for the demonstration of serum antibodies after allogeneic kidney transplantation in two dogs The figures to the left indicate the locations of the test sera on the two donor kidney monolayers No 1 donor 70 No 2 9 recipient 69 tested 0 2 8 17 22 24 26 and 28 days after transplantation No 10 dog anti dog kidney immune serum No 11 donor 67 No 12–18 recipient 68 tested 0 4 7 11 21 28 and 35 days after transplantation Recipient 68 received kidney transplant from donor 67 and recipient 69 from donor 70 Recipient 68 which rejected the kidney graft on day 7 was grafted with skin from donor 67 on day 28

of the antibody titres measured by the mixed haemadsorption technique The positive control was a hyperimmune serum from a dog immunized with allogeneic kidney cells This serum consistently gave a stronger reaction with kidney cells than with bone marrow cells

The kinetics of the antibody response had a different course when donor bone marrow cells were used as target cells (Figs 3 and 4) The curve was biphasic including a transient early response with a peak around day 5 followed by a reduction in titres during the second week after transplantation and a subsequent slow increase over a period of one to two months The time course of this antibody response was rather similar to the one obtained when cytotoxic antibodies against bone marrow cells were measured (Fig 1) The donor serum controls often gave a weak positive haemadsorption reaction particularly when syngeneic bone marrow cells fused with HeLa cells were used (Fig 3) This background activity could be reduced by absorption of the serum with HeLa cells

Fig 5 shows the results obtained by the mixed haemadsorption technique when two recipient sera (no 68 and 69) were tested on kidney

cell monolayers from their respective donors (no 67 and 70) All four dogs were from the same litter It can be seen that recipient no 68 only reacted with the kidney cells from its own donor (no 67) while serum from recipient 69 reacted with none of the donor cell cultures An explanation of the lack of antibody activity in serum samples from the latter recipient was offered by the observation that the grafted kidney never was functioning due to occlusion of the anastomos of the renal artery When a skin graft was performed on recipient 68 four weeks after the kidney transplantation the dog responded with a second set rejection and increased antibody titres This was not observed when recipient no 69 was similarly skin grafted

Heterophile Haemagglutinating Antibodies

Recipient sera were found to be capable of agglutinating rat erythrocytes All dogs tested had pretransplantation titres of haemagglutinins ranging from 50 to 200 Following kidney transplantation the haemagglutinating activity rose rapidly in five out of six recipients reaching titres of 400 to 800 at the time of rejection or slightly thereafter (Fig 6) The haemagglutinin titres were unaffected by the absorption of the recipient sera with allogeneic dog erythrocytes Furthermore donor erythrocytes were not agglutinated by their respective recipient sera (Fig 7) Thus the agglutinins did not represent an iso antibody response to accidentally transplanted donor erythrocytes Neither did the same response represent antibodies against Forssman antigen since the recipient sera had haemagglutinating titres of only 10 or less against

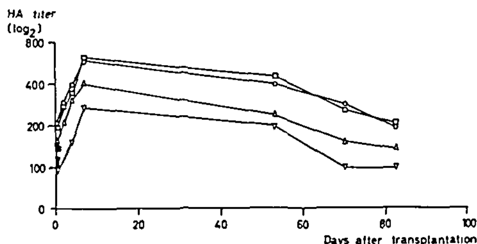


Fig 6

Heterogeneic anti rat haemagglutinin response following renal transplantation No absorption □■ absorption with allogeneic erythrocytes ○● absorption with allogeneic kidney cells ▲ absorption with donor bone marrow cells ▽▼ The filled symbols represent donor sera

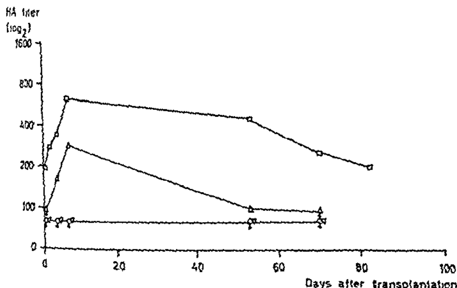


Fig 7

Comparison of heterogeneic haemagglutinin titres in a recipient of a renal allograft. Rat haemagglutinins □ guinea pig haemagglutinins △ sheep haemagglutinins ○ iso agglutinins ▽

sheep erythrocytes and titres of 40 or less against guinea pig erythrocytes while the activity against rat erythrocytes was much stronger (Fig 7). In further tests of the specificity of the produced antibody it was found that absorption of the recipient sera with allogeneic kidney cells significantly affected the agglutinin titres and that absorption of the sera with donor bone marrow cells caused an even more pronounced reduction of the agglutinating activity (Fig 6).

Serum from a dog which had been immunized with allogeneic kidney cells agglutinated rat erythrocytes at a dilution of 900 but had no detectable agglutinating activity against sheep erythrocytes. Absorption of this serum with a pool of allogeneic kidney cells from two dogs reduced the agglutinin titre against rat erythrocytes by about 40 per cent.

Different types of agglutination patterns were often observed with recipient sera collected prior to or immediately after transplantation as compared to sera obtained about one week after transplantation. The former sera gave either a wide diffuse or a compact granular agglutination pattern while only the dense compact type of agglutination was observed with serum samples collected at the time of the antibody rise after transplantation. A similar difference in the agglutination patterns observed between early response and hyperimmune anti BGG and anti BSA sera was described by *Bauer & Stavitsky* (1961). These authors suggested that macroglobulin antibodies present in the early response sera were responsible for the compact granular agglutination

pattern. If this interpretation of their data is correct it is likely that the transplantation response involved primarily IgM antibodies.

DISCUSSION

Altman & Simonsen (1964) demonstrated cytotoxic antibodies in the serum of dogs which had received allogeneic transplants of skin and kidney. Significant cytotoxic activity was observed first about two weeks after grafting and the early antibody peak noted in the present study was not reported. This discrepancy between these results can however be spurious considering that the early antibody rise is hard to detect due to its transient nature and that *Altman & Simonsen* primarily used skin grafting while we used kidney grafts. Also the target cells differed as they used donor leucocytes and we primarily bone marrow and kidney cells. As in *Altman & Simonsen's* study the cytotoxic activity reported here was reduced by absorption with donor cells.

Several investigators (*Medawar* 1946, *Altman* 1963, *Hancock & Mullan* 1962, *Puza et al.* 1964 and *Kuhns et al.* 1964) have described the appearance of haemagglutinins against donor erythrocytes following allografting, but these haemagglutinins were not found to accelerate graft rejection. The recent data by *Iwasaki et al.* (1967) and *Rapaport et al.* (1967) are of greater relevance to the present results. These authors demonstrated humoral antibodies in human recipients after renal homotransplantation. The antibody most extensively studied by *Iwasaki et al.* (1967) reacted with heterogeneic antigen(s) on sheep erythrocytes and was similar to the haemagglutinin described in the present study in at least three respects: 1) it was distinct from the Forssman antibody, 2) it was selectively absorbed by donor cells and 3) as in the present study the kinetics of the appearance of the antibody suggested a response to allograft antigens, a conclusion which was further strengthened by the absorption experiments made in both studies. Serum from their control cases were, however, as a rule negative, whereas *Rapaport et al.* (1967) and we have found most recipients to have low haemagglutinating titres.

The haemagglutinins described in the present study did not represent isoantibodies against donor erythrocytes nor were these heterogeneic antibodies directed against Forssman antigen. The fact that donor cells were capable of absorbing most of the agglutinin activity selectively together with the kinetics of the agglutinin response rather indicated that the antibodies represented a response to allograft antigens. Recently we have extended this assessment of heterogeneic antibodies to human responses to kidney transplants. The preliminary results obtained so far are similar to those reported here for dogs. Thus the human sera contained heterophile haemagglutinating antibodies

against rat cells which were not in the Forssman category and which differed from Paul Bunnell like antibodies and Wassermann antibodies The demonstration of heterophile antibody responses in association with allograft rejection in the study by *Rapaport et al* (1967) and in the present report point to the possible use of this antibody system for detecting lingering transplant rejection crises

A plausible explanation for the presence of the heterogeneic haemagglutinins in both canine and human renal transplantation is that particularly rat erythrocytes possess antigen (s) which cross reacts with dog and human iso antigen(s) present in certain organs e g the kidney Cross reaction between heterogenetic erythrocyte antigens and antigens isolated from horse and guinea pig kidney has earlier been described (*Fuji & Nelson* 1963)

Convincing evidence for the specificity of the antibodies measured in the mixed haemadsorption tests was obtained Thus the recipient sera had little or no antibody activity when reacted with target cells from dogs other then the donor (Fig 5) and donor cells were capable of absorbing the antibodies Also the kinetics for the appearance of these antibodies suggested a response to allograft antigens

The absence of the early antibody peak in the mixed haemadsorption test when kidney cells were target cells was probably not due to the inability of the indicator system to register early (IgM) antibody since also this early phase of the response was seen when bone marrow cells were used A likely explanation is that the early response antibodies were absorbed by the still intact kidney graft and that circulating antibodies became detectable first when antigenic sites in the kidney were saturated or more probably when the kidney circulation was impaired Thus the present data support the findings by *Hager et al* (1964) *Milgrom et al* (1966) and *Williams et al* (1967) which suggest that detection of circulating antibodies in the recipient is hampered by absorption of these antibodies by the functioning kidney transplant It is to be noted that the intact kidney graft was unable effectively to absorb antibodies against bone marrow cells and in addition the dog anti dog kidney serum reacted more strongly with allogeneic kidney cells than with bone marrow cells These findings suggested that the mixed haemadsorption tests measured both organ (kidney) specific antibodies and antibodies against allograft antigens

The clinical significance of the absorption of circulating antibodies in the transplant is difficult to evaluate But in human renal transplants that have rejected later than 11 days after the transplantation *Porter* (1967) found gamma globulin and complement in the arteries arterioles and glomerular capillaries *Jonasson et al* (1967) similarly demonstrated accumulation of gamma globulin in the glomerular capillary loops of human renal transplants In addition immunoglobulin and complement were seen within proliferating endothelial lesions IgM and IgG immunoglobulins have also been demonstrated in the vessel

wall of rejecting unmodified canine transplants (*Horowitz et al* 1961). Lesions of the arteries, arterioles and veins and infiltration of lymphoid cells (*Kountz et al* 1963, *Porter et al* 1964) dominate the initial phase of the rejection process. It is likely that the absorption of circulating antibodies and the formation of antigen-antibody-complement complexes in the vessel wall can initiate and contribute to these vascular lesions as seen in the Arthus reaction.

SUMMARY

Circulating antibodies directed against donor cells were demonstrated following allogeneic kidney transplantation in dogs. A transient weak cytotoxic antibody response occurred 5 to 7 days after transplantation. The kinetics of this response was similar to the antibody response measured by the *mixed haemadsorption technique using donor bone marrow* cells as target cells. In contrast, antibodies against donor kidney cells were demonstrable with the haemadsorption test first when the circulation in the graft was impaired. Removal of the kidney graft caused a rapid increase in the latter type of antibodies. Skin grafting performed subsequent to rejection of a kidney graft resulted in a second set reaction and a rise in antibodies detectable by the mixed haemadsorption test.

Recipient sera contained heterogeneic agglutinins against rat erythrocytes. These agglutinins did not represent antibodies against Forssman antigen or against erythrocyte iso-antigens. The agglutinin titres in most recipient sera increased at the time of rejection and the agglutinins could be partially absorbed by donor cells.

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C REACTIVE PROTEIN IN APPARENTLY HEALTHY INDIVIDUALS (BLOOD DONORS) RELATED TO AGE

By

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Received 9 II 68

A previous study on the presence of C reactive protein (CRP) in the sera from randomly selected men aged fifty showed a total prevalence rate of 84 per cent when the test was performed with a micromodification of the comparative double diffusion in gel technique (7 8) Trace amounts of CRP (< 1 µg/ml serum) were noted in 52 per cent of this series and the presence of CRP at this level seemed to be of minor clinical significance (8)

In contrast to these findings studies on sera from healthy children between 1 and 15 months of age showed a lower prevalence rate of CRP containing sera (12) The intent of the present study was to examine the prevalence rates of sera with different CRP concentrations and their relation to the age of apparently healthy individuals

MATERIAL AND METHODS

Blood sera were obtained from 405 blood donors (343 males and 62 females) The age of the donors varied from 18 to 49 years The sera were collected during November 1964 to February 1965 and were kept frozen at −20 C until the analyses were performed The control of the state of health of the blood donors was limited to anamnestic inquiries with regard to certain diseases namely syphilis malaria tuberculosis hepatitis asthma or other allergic disorders Only individuals free of these diseases are accepted as blood donors The Wassermann test as well as the Meinicke and Kline flocculation reactions were negative in all instances The donors are also obliged to state recent diseases vaccinations etc prior to donation No physical examination of the donors was performed but sedimentation rate and hemoglobin were generally determined

Examination of the sera with regard to the presence of CRP was performed with a micromodification (13) of the double diffusion in gel technique (10) The sensitivity of this technique was about 0 5 µg CRP/ml (7) Quantification of CRP was performed with the halo technique as described by *Mancini Carbonara & Heremans* (5) slightly modified (7) CRP concentrations exceeding about 1 µg/ml could be measured with this technique (7)

The investigation was supported by grants from the *Faculty of Medicine University of Göteborg* and the *Ellen Walter and Lennart Hesselman Foundation for Scientific Research*

The sera were grouped according to their CRP content as follows

Negative Sera without demonstrable CRP as determined with the comparative double diffusion in gel technique (< 0 5 μg CRP/ml)

Trace amounts Sera giving a positive reaction in the double diffusion in gel technique but in which the CRP concentration was too low to be estimated by the halo technique (< 1 μg CRP/ml)

More than 1 μg CRP/ml Sera with quantifiable CRP concentrations were divided into two groups (1–4 2 μg/ml and > 4 2 μg/ml)

The donors were divided according to age into 6 groups each covering 5 years except for the youngest group which was 7 years

For the trend analysis of the possible relation between the prevalence rate of CRP negative sera and age the test for linear trends in proportions and frequencies described by *Armitage* (1) was used

RESULTS

The numbers of women in the different groups were too small to allow statistical analysis in regard to sex and therefore the series was treated as an entity

The prevalence rates of sera with different CRP concentrations related to the age of the blood donors are graphically represented in Fig 1 The prevalence rates of sera with CRP concentrations exceeding 4 2 μg/ml shows no obvious variation over the studied age range There is a decrease in prevalence rates of sera without demonstrable CRP from 49 per cent in the age group 18–24 years to 23 per cent in the age group 45–49 years This decreasing trend is statistically significant (χ^2 = 9 4 1 d f 0 005 > P > 0 001) The increase of the prevalence rates of CRP containing sera with age was noted mainly in the group containing trace amounts but also to some degree in the group containing 1–4 2 μg/ml

The distribution of sedimentation rate according to age is found in Fig 2 In four instances the sedimentation rate exceeded 20 mm/h and in another three instances it was between 16 and 20 mm/h

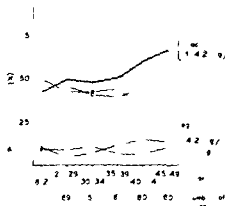


Fig 1

Prevalence rates of sera with different CRP concentrations related to age in a series of 405 blood donors

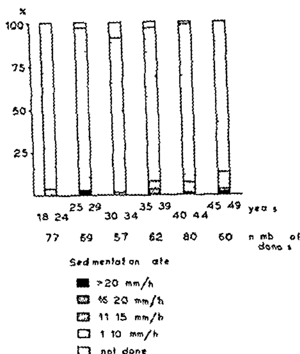


Fig 2

Distribution of erythrocyte sedimentation rates according to age in a series of 40> blood donors

DISCUSSION

CRP is generally thought of as a sensitive sign of an active inflammatory or necrotic process in the individual On the basis of the results of a previous study however it was proposed that the presence of trace amounts ($< 1\ \mu g$ CRP/ml) was probably not indicative of an active CRP stimulating disease process (8) The existence of a relationship between the prevalence rate of CRP containing sera and age was suggested by the results of a preliminary study of blood donors (6) This relationship was supported by the results of studies on healthy children (12) which showed a considerably lower total prevalence rate of CRP containing sera (17 per cent) compared to that found in sera from a random sample of 50 year-old men (84 per cent) (8) A relationship between the incidence of CRP and age has been reported by *Oka & Kylila* (9) for patients with rheumatoid arthritis Similar findings were described by *Hedlund* (3) and *Renger Kanehl & Kanehl* (11) in analyses of patients with different diseases of the liver and biliary tract However these studies dealt with diseased persons and no quantification of the CRP was performed On the other hand *Kettel* (4) using the capillary tube precipitation test in a study of 54 subjects

from an almshouse and with a mean age of about 73 years concluded that age in itself did not influence the outcome of the CRP reaction

The present series of blood donors comprised subjects of different ages Since there are uniform demands regarding the state of health for acceptance as blood donor it might be supposed that the different age groups are comparable with respect to this factor Subjects with a sedimentation rate exceeding 15 mm/h were rather few (7 out of 405) and not confined to any special age group This indicated no preponderance of morbidity in the older age groups The rather constant prevalence rate of sera containing more than 4 2 μg CRP/ml in all the age groups investigated suggests that the incidence of disease conditions which stimulate the formation of CRP did not vary with age in the investigated material This finding is also consistent with the presumed similarity in state of health of blood donors of different ages

The aforementioned studies of *Hedlund* (3) *Oka & Kytila* (9) and *Renger et al* (11) dealt with diseased subjects and their findings can probably be ascribed to a greater morbidity with increasing age The increasing prevalence rate demonstrated in this study seems to be limited to the age groups having from trace amounts up to about 4 μg CRP/ml and would generally not have been demonstrated by the techniques used by the aforementioned authors

As was discussed in the previous study of 50 year old men the presence of trace amounts of CRP probably does not reflect a concomitant CRP stimulating disease (8) A rising prevalence rate of sera containing low concentrations of CRP (trace amounts up to about 4 μg/ml) in relation to age was observed in the present study It seems feasible to assume that this relationship may reflect changes associated with aging In this connection it is interesting to note that *Bottiger & Svedberg* (2) recently demonstrated that there is a significant increase of the erythrocyte sedimentation rate with increasing age in healthy subjects

The described findings raise the question of the normal limit for blood serum CRP Authors hitherto concerned with this question agree that CRP is no normal component of the blood Considering the present findings this view should be modified *It is evident that CRP to a certain degree* depending upon the age of the individual can be demonstrated in low concentrations in normal subjects that is in subjects without clinically demonstrable disease However to elucidate whether a positive finding within the low range in a single specimen is an indication of incipient or declining disease or can be considered normal a follow up testing of the individual is necessary

SUMMARY

The CRP content has been determined in blood sera from 405 blood donors by means of immunodiffusion techniques and the prevalence

of sera containing various CRP concentrations has been related to the age of the donors There was a rather constant prevalence rate of sera containing more than 4 2 μg/ml in the different age groups whereas an increase in prevalence rate of sera with CRP concentrations below 4 2 μg/ml was observed with increasing age It is suggested that the observed presence of low concentrations of CRP (from trace amounts up to 4 μg/ml) might reflect changes associated with aging

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NON-SPECIFIC ADSORPTION OF FITC-LABELLED SERUM GLOBULINS TO *STAPHYLOCOCCUS AUREUS*

By

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When working on the identification of *Neisseria gonorrhoeae* (*N. gonorrhoeae*) by means of the fluorescent antibody technique, cross-reaction with antigenically related *Neisseria* species did not present any difficulties. However, certain strains of *Staphylococcus aureus* (*S. aureus*) were stained brilliantly by rabbit antigonococcal sera labelled with fluorescein isothiocyanate (FITC) and in addition they exhibited a striking morphological similarity to gonococci (*13*). It was thought originally either that "natural" antibodies or mutual antigens were responsible for this non-specific staining. The first experiments to be described were performed in order to determine which of these explanations might be correct. The results obtained suggested a non-immunological affinity of certain strains of *S. aureus* for serum globulins, and the later experiments included an investigation of this phenomenon.

MATERIALS AND METHODS

Bacterial Strains

S. aureus: One hundred and nineteen strains were kindly supplied by Dr. *Kirsten Rosenthal*, Department of Diagnostic Bacteriology, Statens Seruminstitut. In addition *S. aureus* Cowan type I (*NCTC 8530*), Smith's strain (*NCTC 10399*) and the strain Wood 46 (NCTC 10344) were included.

S. albus: One hundred and one strains were isolated from specimens received for identification of *N. gonorrhoeae*. The following criteria were used for classification: —On solid medium the strain should grow with circular, white or whitish colonies with a smooth, shiny surface. It should form no coagulase and should appear as Gram-positive cocci in grape-like clusters or small groups in Gram-stained smears from broth cultures. Differentiation between *S. albus* and *Micrococcus* was not attempted.

N. gonorrhoeae: Freshly isolated strains identified by the routine procedure of the Neisseria Department (?) were used as controls in experiments with labelled antigonococcal sera.

Media

Broth: Test medium broth was made according to the method of [illegible] & *Smith* (*20*).
Ascitic broth was made by the addition of [illegible]5 per cent ascitic fluid to beef broth.
Blood agar was made from the peptone agar containing 10 per cent horse blood.
Ascitic agar was made from the peptone agar containing 30 per cent ascitic fluid.
Chocolate ascitic fluid agar: The preparation of this medium was described by

A. Reyn (92, pp. 462–464). Squibb Mycostatin® was added to inhibit the growth of moulds (25–30 units per ml substrate).

Sera

Normal sera. Rabbits with a negative gonococcal complement fixation test (11) were selected and used as source of normal rabbit sera and for the immunization with gonococcal and staphylococcal antigens. Pre-immunization samples of serum were collected from all animals. Sera from non-immunized guinea pigs, rats and sheep were obtained from the Institute's own colony of conventional animals.

Rabbit antistaphylococcal sera. Ten selected strains of S. aureus (vide infra) were used for preparation of antigens.

I) In order to include all more or less labile cell wall substances and extracellular products of the bacteria, the strains were grown in broth. When the density of the culture was about 10^9 bacteria per ml, formalin was added to a final concentration of 0.5 per cent. One pool was made from five strains showing non-specific staining by the fluorescent antibody test (FAT) and another from the five strains that did not exhibit non-specific staining reactions. These two antigens were each injected intravenously into groups of four rabbits. The injections were given twice a week for three weeks with increasing doses of antigen, viz. 0.1, 0.25, 0.50, 1.0, 2.0 and 2.0 ml. Blood samples were collected by heart puncture nine days after the last injection. Three weeks later a booster injection of 1.0 ml was given intravenously, and after a further six days the animals were exsanguinated. The controls were two rabbits immunized with formalin-treated broth according to the same schedule, and two non-immunized rabbits from which blood samples were collected at the same intervals.

II) In order to prepare somatic antigens from the same ten strains of S. aureus, 18-hour cultures were harvested from blood agar in phosphate buffered saline, pH 7.2 (PBS) (13), and autoclaved at 120° C for two hours and a half. The suspensions were centrifuged, washed three times in PBS and resuspended in PBS containing 0.3 per cent formalin. Before immunization the density of the suspensions was adjusted to that of a staphylococcal suspension of 2×10^9 living cells per ml. The suspensions were pooled according to strains as described under I. The immunization schedule was that used for gonococcal antigens (13).

III) A pool of ten non-specifically reacting strains of S. aureus (including the five used under I and II) were grown on blood agar and harvested in PBS containing 3 per cent formalin as described for gonococcal antigens. The immunization schedule was that used for gonococcal antigens.

Rabbit antigonococcal sera. Details of the preparation of antigen and the schedule for immunization have been published earlier (13). Antisera against boiled and autoclaved gonococcal antigen were produced in the same way. Gonococcal strains grown in ascitic broth were treated with formalin and the immunization was performed as described under *Rabbit antistaphylococcal sera I*.

Fluorescent Antibody Test (FAT)

The labelling of sera with FITC and the performance of the test have been described earlier (13).

Reading.

++++ denotes a brilliant yellow-green fluorescent layer covering uniformly the surface of each bacterial cell.

+, ++ and +++ indicate increasing degrees of homogeneity and intensity of the fluorescence of a bacterial population.

0 denotes a pale bluish fluorescence.

Gonococcal Complement Fixation Test

The test was performed according to M. Kristensen (11). The results are given as log₂ to the reciprocal value of a serum dilution giving 50 per cent haemolysis.

Indirect Haemagglutination (IHA) Test for Demonstration of Staphylococcal Antibodies

In general the test was performed as described by Nelson & Gorzynski in 19[illegible]9 (17). The antigen employed was a saline suspension of bacteria that had been grown for

18 hours on blood agar media. The density of the suspension was adjusted to 20×10^9 bacteria per ml. This suspension was kept at −20° C. The antigen modification and the enzyme treatment of sheep red blood cells (SRC) were carried out simultaneously, i.e. a 4 per cent suspension of washed SRC in saline was mixed with an equal volume of antigen diluted in PBS pH 7.38 containing 50 mg trypsin per litre (Difco trypsin 1:250). The mixture was incubated in a water bath at 37° C for 1 hour, then washed three times in saline and resuspended to form a 2 per cent suspension of SRC in saline. SRC treated in the same way with trypsin but without antigen were used as controls.

Sera were inactivated by heating at 56° C for 30 minutes and absorbed with an equal volume of packed SRC. The absorption was performed with a 1:10 dilution of serum in physiological saline for 10 minutes at room temperature. Serial dilutions with 0.3 ml volumes were made in plastic haemagglutination trays ("Linbro"). One drop (about 0.05 ml) of antigen sensitized SRC was added to each cup. As controls the two highest concentrations of each serum were tested against non sensitized SRC. Furthermore, both sensitized and non sensitized SRC were tested in the diluting medium (physiological saline). The trays were shaken carefully and left overnight at room temperature.

The reading of the agglutination pattern was graded from ++++ to 0. The +++ and ++++ indicated strongly positive reactions in which the even layer of SRC had rolled down from the edges of the cup. A ++ reaction showed an even layer of SRC covering the bottom of the cup. Formation of a big ring was recorded as + and a very small ring or a spot in the bottom of the cup as 0. In preliminary experiments the antigen dilution was adjusted to give a clear cut endpoint. Usually a dilution of 1:10 was employed. The results was recorded as $\log_{10}$ to the reciprocal value of the lowest serum concentration giving + reaction.

In the IHA inhibition experiments each serum was diluted serially in two parallel rows. One drop of the inhibitor was added to each cup in the first row and one drop of saline to each cup in the corresponding control row. The trays were left for two hours at room temperature before SRC were added as described above.

Double Diffusion in Agar (Ouchterlony Analysis)

The macro technique described by *Ouchterlony* was used (18), except that the agar was 1 per cent Reinagar Behringwerke® in phosphate buffer containing 0.65 g of sodium azide per litre and 1.12 g of EDTA per litre (EDTA, Merck Titriplex® III).

RESULTS

Staphylococci which stained non specifically were detected in the first experimental series employing FAT for the diagnosis of gonorrhoea. The question arose: how often do such strains occur in specimens received for identification of *N. gonorrhoeae*. In the remaining part of that experiment attention was therefore paid to isolating colonies resembling staphylococci. The strains isolated were examined by FAT with an antigonococcal conjugate and subcultured for identification by the criteria mentioned in *Materials and Methods*. After isolation of 101 strains, all belonging to the genus *Staphylococcus albus* and all unstainable in FAT, a number of identified *S. aureus* strains were obtained from Dr. *K. Rosendal*[1].

Among 113 strains of *S. aureus* examined by FAT with FITC labelled antigonococcal globulin 10 per cent showed brilliant staining with the characteristic appearance of a specific staining reaction, i.e. a fluorescent capsule like layer covering uniformly the surface of each bacterial

[1] Department of Diagnostic Bacteriology, Statens Seruminstitut, Copenhagen.

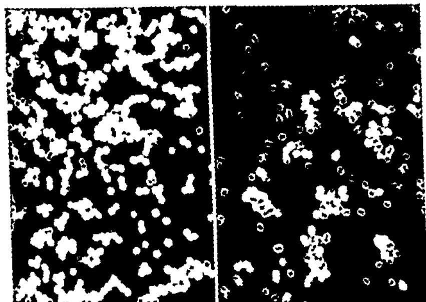


Fig 1

Left S *aureus* Cowan type 1 stained with FITC labelled rabbit antigonococcal globulin Right S *gonorrhoeae* stained with same conjugate Magnification 1500 Film Kodak Tri X Exposure time 1 minute

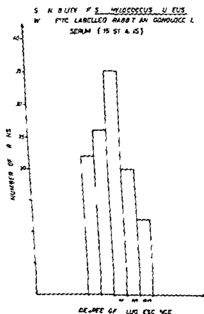


Fig 2

cell (Fig 1) The distribution of the strains according to stainability is shown in Fig 2 The groups showing 0 and + + + + fluorescence were most easily recognized and the results were highly reproducible In the intermediate groups the results were subject to minor variations depending on the conjugate employed Fifty two of the 115 strains were simultaneously tested with a conjugate prepared by the author and a Bacto FA *N gonorrhoeae* preparation The two sets of results were identical

TABLE 1
Staining Titre of FITC Labelled Rabbit Antigonococcal Globulin

Strains used as antigen	Dilution of conjugate					
	Nil	1 20	1 40	1 80	1 160	1 320
N gonorrhoeae	++++	+++	++	++	+	+
S aureus 8341	++++	++++	++++	++++	++++	++++
S aureus 20036	0	0	0	0	0	0

+ + + + → 0 denotes the degree of fluorescence (see text)

Serial dilutions of labelled antigonococcal globulins were tested in IAF with *N gonorrhoeae* and *S aureus* as antigens Several conjugates were tested against various gonococcal and staphylococcal strains In all cases the pattern was as shown in Table 1 In the known specific antigen antibody reaction a + + + + reaction was found in dilutions up to 1 40 when a dye/protein ratio of 1 40 had been used during the labelling procedure Invariably the reactivity between the antigonococcal conjugate and a cross reacting *S aureus* was much stronger A + + + reaction was often still found at a dilution of 1 1280

It was concluded from these experiments that staphylococci which stained non specifically occurred so often that they could not be ignored as a possible risk in the diagnosis of gonorrhoea by IAT

With a view to investigating the phenomenon in greater detail five strains of *S aureus* showing strong (+ + + +) cross reactivity by IAT and five which were non reactive were examined other characteristics of the ten strains obtained from *K Rosendal* (23) are given in Table 2

Attempts to Demonstrate Antigenic Relationship between N gonorrhoeae *and* S aureus

The immunization schedules used were compiled with a view to obtaining as much information as possible at a time Primarily the intention was to examine the concept of *Deacon et al* (5) that only sera containing antibodies to a presumed labile surface antigen of virulent gonococci would react by IAT and this determined the schedules used for production of antisera to gonococcal antigens Antisera to

TABLL 2
Strains of S aureus Selected for Further Examination

S *aureus* No /1961 62	Phage type	Production of lipase	Sensitivity to antibiotics	FAT§
8341	83A	0	I ST	++++
10476	83A	0	PST	++++
107?7	NT u	0	I ST	++++
19968	3A	+	P	++++
20004	52/5?A/80/81	0	Sensitive	++++
1?466	3C/55 u	+	Sensitive	0
19910	83A u	0	PST	0
199?0	83A	+	PS	0
20001	71	+	P	0
20036	3B/3C/71	+	I	0

Results obtained by A *Rosendal* for methods of *ref* (23)
Capitals indi ate resistance to penicillin (I) streptomycin (S) and tetracyclines (T)
§ Stainability with FITC-labelled rabbit antigono occal globulin

TABLE 3
Comparison of Serum Antibody Levels in Rabbits Immuni ed Against Gonococcal and Staphylococcal Antigens

Antiserum against	Number of rabbits	Indirect staphylococcal haemagglutination test (log titre mean value) with antigen from			Gonococcal complement fixation test $\log_{10}$ titre mean value
		reactive *strains*	non reactive strains	mixed *strains*	
Gonococcal antigen treated with					
a) 3% formalin	11	< 1 30	< 1 30	< 1 30	3 31
b) heat (100 C 30 min)	4	< 1 30	< 1 30	< 1 30	3 03
c) heat (120 C 2¼ hrs)	4	< 1 0	< 1 30	< 1 30	(1 60)
d) 0.5% f rmalin (broth culture)	3	< 1 30	< 1 30	< 1 30	3 04
aphylococcal antigen treated with					
a) 3 formalin					
1) reactive strains	29	3 37a	3 15§	3 81	< 0 60
b) heat (120 C, ? n hr					
1) reactive strain	3	? 91	2.51	3 40	< 0 60
2) non reactive strains	4	2 58	2 85	3 78	< 0 60
c) 0 5% formalin (broth ulture)					
1) reactive stra n	4	3 70	3 70	3 93	< 0 60
2) non reactive train	3	3 93	4 15	4 15	< 0 60

In these sera high level of gonoc ccal antibodies det ctable by means of a gono coccal haemolysis test (2)
§ Only 6 sera examined
Reactive and non rea ti e staphylococcal strains see explanation in text under *Discussion*

cross reacting staphylococci were produced at the same time. Treatment similar to that used for gonococcal antigens was carried out in order to include detection of cross reaction both with heat stable and heat labile antigens. Similar antigens and antisera were prepared from staphylococci that did not cross react by FAT. As shown in Table 3 antisera against gonococcal antigens did not contain demonstrable antibodies against staphylococcal antigens. Similarly antisera to staphylococcal antigens did not react with gonococcal antigens. Pre immunization sera from all the rabbits were negative in both tests.

A highly sensitive method for the detection of cross reactivity is the IHA inhibition test. It was found in preliminary experiments that inhibition corresponding to one two fold dilution step in the staphylococcal IHA test was produced by addition of one drop of a 1:1000 dilution of the sensitizing antigen. Gonococcal antigens (heat treated at 56 C for 20 minutes, at 100 C for 30 minutes or at 120 C for two hours and a half) did not inhibit the reaction between staphylococcal antigen and the homologous antibody.

TABLE 4

Stainability of S. aureus with Various FITC Labelled Rabbit Antigonococcal Globulins All Diluted 1:10

S. aureus strains	Antisera against *N. gonorrhoeae*						
	Strain 11413 1940	Strain 19397 1940	Cells of strains treated with: 37 C	100 C 30 min	120 C 2 hrs	0.5% F‡ (br th)	Bacto FA *N. gonorrhoeae*
8341	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
10176	+ + +	(0)	(0)	(0)	(0)	(0)	+ + + +
10 27	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
19958	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
20004	+ + +	+ + +	+ + +	+ + +	+ + +	+ + + +	+ + + +
12466	0	0	0	0	0	0	0
19910	0	0	0	0	0	0	0
19920	0	0	0	0	0	0	0
20001	0	0	0	0	0	0	0
20036	0	0	0	0	0	0	0
N. gonorrhoeae control	+ + + +	+ + + +	+ + + +	+	+ + + +	+ + + +	+ + + +

‡ Formalin
§ See text

Various antigonococcal sera were labelled with FITC and used by FAT against the ten selected staphylococcal strains as antigens (Table 4). All conjugates showed cross reactivity with the same four (five) staphylococcal strains and the remaining strains were never stained. The conjugates examined included a commercial one (Bacto FA *N. gonorrhoeae*) and two prepared from antisera against antigenically

TABLE 5

Stainability of S aureus with FITC Labelled Globulins from Pre Immune and Immune Rabbits All Conjugates Diluted 1 10

S aureus strains	Rabbit 9801		Rabbit 9813		Rabbit 9833		Rabbit 9843	
	P	I	P	I	P	I	P	I
8341	++++	++++	++++	++	++++	++++	++++	++++
10476	(0)	(0)	(0)	(0)	(0)	(0)	(0)	++
10727	++++	++++	++++	+++	++++	++++	++++	++++
19968	++++	++++	++++	+++	++++	++++	++++	++++
20004	++	+++	++++	++	+++	++++	++	++++
19477	0	0	0	0	0	0	0	++
19910	0	0	0	0	0	0	0	++
199 0	0	0	0	0	0	0	0	++
20001	0	0	0	0	0	0	0	++
20036	0	0	0	0	0	0	0	+
N gonorrhoeae	0	++++	0	++++	0	++++	0	0

Rabbits 9801 9813 and 9833 were immunized with gonococcal antigens
rabbit 9843 with a staphylococcal antigen
P pre immune
I immune
See text

well known gonococcal strains viz N gonorrhoeae 11413/1940 and 10927/1940 (21) One strain S aureus 10476 was outstanding in that the factor responsible for the stainability was lost after about six months The original strain had been kept lyophilized and was now retested Microscopy showed a characteristic mixture of brilliantly stained and unstained bacteria with the latter in the majority In Tables 4 5 and 6 such results are indicated by a zero in brackets It was not possible to seperate two different bacterial populations and in sub cultures the dominance of non reactive bacteria was increasing This finding was in contrast to that of all other strains the stainability of which was unchanged after numerous passages

FITC labelled antistaphylococcal sera did not stain N gonorrhoeae

Attempts to Demonstrate Natural Antibodies to S aureus

In the event of non specific staining of S aureus by FITC labelled antigonococcal sera being caused by simultaneously labelled previously formed or natural antibodies to staphylococci it might be possible to select rabbits without or with a sufficiently low serum level of these antibodies

As a first step towards this attempts were made to develop a test for the detection of circulating staphylococcal antibodies On the basis of long experience with the complement fixation test various staphylococcal antigens were subjected to variations of that procedure but reproducible results could not be obtained

that staphylococcal antibodies occur normally in human sera (9), in sera from non-immunized specific pathogen free rabbits and in sera from germ free and normal mice (3, 4).

The aim of the present study was to investigate this broad reactivity of certain strains of *S. aureus* especially with respect to the strong reaction found with FITC labelled rabbit antigonococcal globulin.

The results of the experiments do not support the hypothesis of a possible existence of a hitherto unrecognized antigenic relationship between staphylococci and gonococci. This conclusion is drawn from the following findings. 1) Sera from animals hyperimmunized against gonococcal antigens did not contain antibodies to staphylococcal antigens and *vice versa*, sera from animals hyperimmunized against staphylococcal antigens did not contain gonococcal antibodies. 2) Various gonococcal antigens were not able to inhibit the specific reaction between staphylococcal antigen and homologous antisera in the IHA test. 3) When FITC-labelled sera from normal and immune rabbits were tested with ten selected strains of *S. aureus* as antigens, the reactivity was uniformly linked to the same strains. This suggested that the stainability reflected a characteristic on the bacterial surface and not some factor(s) in the sera tested. 4) FITC-labelled globulins from other species (Table 6) showed high affinity to exactly the same strains.

The claim that "natural antibodies" to staphylococci should cause the stainability of the reactive staphylococcal strains would imply a uniformly high level of *antibodies* to such strains in all sera examined. This assumption seems unreasonable.

Thus the terms *reactive* and *non reactive* staphylococci have been introduced and used in further experiments (14, 15).

Specific antigen-antibody reactions and the reactivity of staphylococcal strains were visualized using ferritin labelled globulins and examination by electron microscopy. This technique revealed a picture of two quantitatively different affinities between the globulins and the surface of the bacterial cells (14).

To obtain additional information about non-specific adsorption of globulins to certain strains of *S. aureus* a series of absorption experiments were performed. The results are described in the subsequent paper (15).

Recently a Swedish group of workers have presented strong evidence that the antigen "protein A" from *S. aureus* possesses a non-specific affinity to human and rabbit γG globulins (6, 7). Presumably this finding reflects the same phenomenon as that described for reactive staphylococcal strains. If so, the reactive strains must be strains with high content of "protein A". So far one strain known to contain protein A (Cowan type I (9, 16)) was found typical and strongly reactive and *Wood 46* which does not possess this antigen was non-reactive. It might be added that the capsulated Smith's strain was non-reactive.

Since "protein A" has been shown to be both an agglutinogen and a

precipitinogen and furthermore, to be identical with a precipitating and agglutinating factor in *Jensen's* antigen A (12 24) this strong and non specific affinity might interfere with specific serological tests for staphylococcal antibodies For instance the precipitation of guinea pig globulin also described by *Lofkvist* (16) might be the explanation of the great difficulties encountered in establishing a complement fixation reaction with guinea pig serum as source of complement

The difficulties in obtaining specific especially type specific agglutination reactions in staphylococcal serology has recently been reviewed by *Haukenes* (8) He recommends the use of mannitol salt agar medium for culturing strains to be tested serologically He suggest that strains growing on high salt concentration do not develop some protein layers on the bacterial surface and therefore the reactions with the underlying antigens are not blocked Preliminary experiments in which reactive staphylococcal strains were grown on the mannitol salt agar medium recommended confirmed the suggestion put forward by *Haukenes* All the reactive strains became non reactive thus indicating a loss of protein A

SUMMARY

An explanation of the non specific staining of certain strains of *S aureus* by FITC labelled globulins from normal and immune rabbits has been sought using various immunological methods The results indicate strongly that the reaction between these strains of *S aureus* and globulins is not a specific antigen antibody reaction but a non immunological affinity of the bacterial surface to globulins A possible correlation between the stainability of the strains and their content of the antigen protein A is discussed

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FURTHER INVESTIGATION OF SPECIFIC AND NON SPECIFIC ADSORPTION OF SERUM GLOBULINS TO *STAPHYLOCOCCUS AUREUS*

By

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Received 6 I 68

The use of Coons immunofluorescence technique in bacteriological diagnosis has revealed that certain strains of *Staphylococcus aureus* (*S aureus*) are always stained by sera and globulins labelled with fluorescein isothiocyanate (FITC) (1 2 12 13) Electron microscopy of staphylococci after incubation with ferritin labelled globulins indicated that this phenomenon was due to an adsorption of globulins to the surface of the bacterial cells and furthermore that the binding was not specific in the sense of an antigen antibody reaction (11) The present experiments were performed to elucidate whether serum proteins in general were involved or only e g the γG globulins

Strains that show brilliant non specific staining in the fluorescent antibody test (F A T) are referred to as *reactive* and those reacting only with their corresponding antibodies as *non reactive* strains The four strains of *S aureus* which were examined by electron microscopy (11) were included in the absorption experiments

MATERIALS AND METHODS

Bacterial Strains

Three reactive strains (8341/61-62 4972/62-63 and E [illegible]8/62-63) and two non reactive strains (20036/61-62 and F 1369/62-63) of *S aureus* were used (13) as well as *S aureus Cowan type I* (NCTC 8530) and one strain of *Neisseria gonorrhoeae* (*N gonorrhoeae*) (17924/63-64)

Preparation of Bacteria for Absorption

The staphylococcal strains were grown for 18–20 hours at 36 C on plain agar medium containing 10 per cent horse blood The bacteria were harvested in phosphate buffered sal[illegible] (PBS) pH 7 2 containing 3 per cent formalin The suspension was left for 30 minutes at room temperature washed three times with PBS resuspended in a small amount of PBS and stored at — 0 C Immediately before use the bacteria were [illegible]sned and packed by centrifugation

In one experim[illegible] with tra[illegible]n S *aureus* 497?/6?-63 the concentration and the period of treatm[illegible]nt [illegible]ith formalin were varied 1) 0 5 per cent formalin for 3 hours 2) 3 per cent fo[illegible]n for ½ hour (as above) and 3) 5 per cent formalin for 24 hours All suspensio[illegible] were left at room temperature and finally washed as described above

The gonococcal strain was grown for 16–18 hours at 37° C in a moist atmosphere containing 10 per cent carbon dioxide. The medium was the fermentation medium described by Reyn (19) except that sugar, phenol red and co-carboxylase were omitted. The bacteria were harvested and treated as described for the staphylococcal strain.

Sera

The antigens used for preparation of rabbit antistaphylococcal sera were broth cultures of S. aureus grown to a density of 10^9 bacteria per ml. Formalin was added to a final concentration of 0.5 per cent. Groups of four rabbits were immunized by intravenous injections of increasing doses twice a week for three weeks (doses 0.1, 0.25, 0.5, 1.0, 2.0, 2.0 ml). Blood samples were collected by heart puncture 9 days after the last injection. Serum was separated from the clot and kept at −20° C.

Preparation of rabbit antigonococcal sera has been described previously (12).

For the absorption experiments sera from four rabbits of each group were pooled. Pre-immunization samples of sera from the same rabbits were pooled and served as "normal rabbit serum" (NRS). Equal volumes of serum from 24 healthy donors were pooled to serve as normal human serum (NHS).

Procedure for Absorption of Sera

Undiluted serum was mixed with an equal volume of packed bacterial cells and incubated at 37° C for 45 minutes. The mixture was then centrifuged at 1500 g for 30 minutes. One half of the supernatant was kept for analysis and the other half was absorbed once more with an equal volume of bacterial cells. In one experiment the serum was absorbed three times.

Immunoelectrophoresis

The micromethod of Scheidegger (20) was used with minor modifications. The gel consisted of 1.5 per cent Special Noble Agar (Difco) in sodium barbiturate buffer with calcium lactate, pH 8.6 (7). After application of the sample 4.5–5.0 volts per cm were applied for 70 minutes. Antiserum was added and the plates were left for 45–48 hours at room temperature for diffusion. The samples of rabbit serum were analysed with sheep antiserum against rabbit globulin (Sylvana). The samples of NHS were tested with rabbit antisera against human serum and against human γG globulin. The washed and dried plates were stained with Amido black 10 B.

Agar Gel Electrophoresis

Agar gel electrophoresis (21) was performed using the same buffered agar as that used for immunoelectrophoresis. A mixture of 50 ml of glacial acetic acid, 100 ml of 96 per cent ethanol and 850 ml of demineralized water was used as fixative.

Double Diffusion in Agar (Ouchterlony Analysis) (17)

The agar gel had the same composition as that used for immunoelectrophoresis. A standard punch (LKB) for rosettes with 6 and 8 peripheral wells was used. The absorbed rabbit serum and a two-fold serial dilution of the corresponding unabsorbed serum were precipitated with a sheep anti-rabbit γG globulin (Mann). In the analysis of NHS rabbit antisera against the following human serum proteins were used: albumin, γG globulin, γA globulin and γM globulin.

Determination of Protein Concentrations

The concentration of proteins was determined by Lowry's method (14) or with a TS refractometer (American Optical Company).

Indirect Staphylococcal Haemagglutination Test

The method was described by Neter et al. in 1959 (16). The antigen employed was a saline suspension of 10 living strains of S. aureus cultured on agar. Sheep red cells were treated with trypsin as described in (13).

Gonococcal Complement Fixation Test

The performance of the test and the expression of the results in degrees of potency were as described by Kristensen (9).

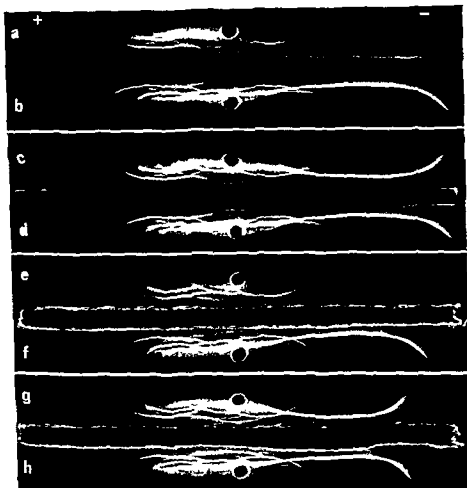


Fig 1

Immunoelectrophoretic analysis of rabbit antistaphylococcal serum absorbed twice with the reactive *S aureus* 4972 (a) absorbed twice with the non reactive *S aureus* 20036 (c) and before absorption (b and d) rabbit antigonococcal serum absorbed twice with the reactive *S aureus* 4972 (e) absorbed twice with *N gonorrhoeae* 17924 (g) and before absorption (f and h) All samples were tested with sheep antiserum against rabbit serum globulins

RESULTS

Absorption of Rabbit Sera

Three samples of each serum pool were absorbed twice with a reactive staphylococcal strain a non reactive staphylococcal strain and the gonococcal strain respectively The qualitative composition of the sera was examined before and after each absorption by immunoelectrophoretic analysis agar gel electrophoresis and double diffusion in agar The absorptions resulted in characteristic changes in the content of γG globulins

Qualitative changes in protein content After one absorption with a reactive staphylococcal strain immunoelectrophoretic analysis of the serum showed a considerable decrease in the content of γG globulin After two absorptions only traces of γG globulin were detectable when the reactive strain 8341/61–62 was used whereas no γG globulin was demonstrable when the more strongly reactive strain 4972/62–63 was used (Fig 1 a) A minor decrease in other globulin components could not be totally excluded After absorption no qualitative differences were demonstrated between the electrophoretic patterns of the normal the antistaphylococcal and the antigonococcal serum

Absorption with non reactive staphylococci or gonococci caused a slight decrease in the globulin content of all the sera investigated (Fig 1 c and 1 g)

Quantitative changes in protein content A quantitative estimate was made from the results of agar gel electrophoresis and double diffusion in agar Using the latter method comparison of each absorbed sample with a two fold serial dilution of the corresponding unabsorbed serum showed that two absorptions with a reactive strain caused a reduction of γG globulin to less than 1/16 of the original content Absorption with a non reactive staphylococcal strain or a gonococcal strain caused a slight decrease in protein content in all cases the γG globulin concentration of the absorbed sample was found to resemble that of the unabsorbed serum more than that of the first dilution (*i e* 1 2) The results obtained by agar gel electrophoresis confirmed these findings

Changes in antibody content Samples in which a sufficient amount of serum remained after the above analyses had been made were tested for content of antibodies to staphylococci and/or gonococci (Tables 1 and 2) Each staphylococcal strain whether reactive or non reactive absorbed all homologous antibody in one step A gradual decrease in titre to gonococcal antigen was found after absorption with a reactive staphylococcal strain but not after absorption with a non reactive strain All homologous antibody was absorbed from the antigonococcal sera by the strain *N gonorrhoeae* 17924

Absorption of NHS

Two sets of experiments were carried out with NHS

The aim of the first series was to elucidate whether a reactive staphylococcal strain was able to absorb human γG globulin as selectively as rabbit γG globulin The following strains of *S aureus* were used 4972/62 63 (reactive) E 228/62 63 (reactive) 20036/61–62 (non reactive) and NCTC 8530 Cowan type I The latter was included on account of its high content of "Protein A (8 15) an antigen which is able to precipitate about 45 per cent of a pooled normal human γG globulin preparation (5) Four samples of NHS were absorbed twice each with one of the four individual strains Before and after each absorption the

TABLE 1

Indirect Staphylococcal Haemagglutination Test

Serum	Titre before abs	Titre after absorption with strains of S aureus						
		8341 × 1	8341 × 2	4972 × 1	4972 × 2	E1369 × 1	E1369 × 2	20036 × 1
Pre immunization (rabbits 9943–50)	<20	<20	<20	–	–	–	–	–
Post immunization with 5 strains of *S aureus* (rabbits 9947–50)	40 000	<20	<20	<20	<20	<20	<20	<20

Antisera to *N gonorrhoeae* were negative (<20)
Reactive strains *S aureus* 8341 and 4972 Non reactive strains *S aureus* E1369 and 20036
– = not examined

TABLE 2

Gonococcal Complement Fixation Test

Serum	Absorbed with		Per cent haemolysis at serum dilution 1/12	1/36	1/108	1/324	1/972	1/2916	Degree of potency
Antiserum to *N gonorrhoeae* 17924	Nil		0	0	0	20	80	100	11
	N gonorrhoea	17924 × 1	80	100	100	100	100	100	0
	N gonorrhoeae	17924 × 2	100	100	100	100	100	100	0
	S aureus	4972 × 1	0	20	80	100	100	100	5
	S aureus	4972 × 2	0	80	100	100	100	100	2
Antiserum to a pool of strains of *N gonorrhoeae*	Nil		0	0	0	20	100	100	11
	S aureus	4972 × 1	40	40	80	100	100	100	(4)
	S aureus	4972 × 2	100	100	100	100	100	100	0
	S aureus	E1369 × 1	0	0	0	20	100	100	11
	S aureus	20036 × 1	0	0	0	20	90	100	11

Neither serum controls nor antigen control showed anticomplementary effect
Normal rabbit sera and antistaphylococcal sera were negative
Reactive strain *S aureus* 4972 Non reactive strains *S aureus* E1369 and 20036

samples were analysed as described under Materials and Methods (Fig 2) Absorption with the non reactive strain caused insignificant changes for γG globulin whereas absorption with the reactive strains left only 1/10 to 1/20 of the γG globulin after two absorptions The contents of albumin γM and γA globulin as estimated by Ouchterlony analysis were only slightly changed after absorption with the four strains mentioned

The absorption capacity of *S aureus* Cowan I was found to be similar to that of known reactive strains

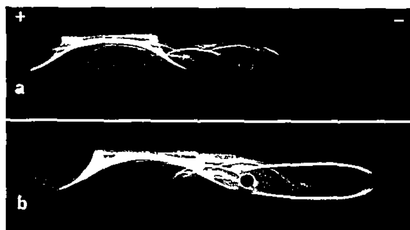


Fig 2

Immunoelectrophoretic analysis of normal human serum absorbed twice with the reactive *S aureus* F 228 (a) and the non reactive *S aureus* 20036 (b) On both slides the upper trough contained rabbit antiserum against human serum and the lower trough rabbit antiserum against human γG globulin The elongation of some of the precipitation lines is due to reactions between excess of antigens used for absorption of the anti human γG globulin and the anti human serum

The second set of experiments was prompted by the findings of *Prochazka* (18) who demonstrated that prolonged treatment with a strong formalin solution eliminated the non specific staining of *S aureus* in I A T *S aureus* 4972 (reactive) was treated with varying concentrations of formalin for different periods as described under Materials and Methods The two suspensions treated with 0.5 per cent and 3 per cent formalin showed selective absorption of γG globulins as found earlier In contrast the suspension treated with 5 per cent formalin for 24 hours also absorbed γA and γM globulins and furthermore the residual γG globulin content of the samples was approximately two times higher than after absorption with the two former suspensions

DISCUSSION

Earlier findings indicated that the non specific staining of certain strains of *S aureus* in the I A T was due to a non immunological affinity to rabbit serum globulins (11 13) Other explanations of the phenomenon as antigenic relationship or natural antibodies were thought to be less probable

The present experiments show that this affinity is predominantly limited to γG globulins A reactive strain absorbed all γG globulins including homologous antibodies whereas a non reactive absorbed all homologous antibody without any significant decrease in γG globulin content These differences indicate that two mechanisms are involved

Another antigen antibody system viz a gonococcal strain its homologous antiserum and a polyvalent gonococcal antiserum was included

in the absorption experiments Again the changes caused by absorption with a reactive staphylococcal strain were clearly distinguishable from those found in the other samples The gonococcal strain absorbed readily all homologous antibody without demonstrable changes in globulin content whereas absorption with the reactive staphylococcal strain caused a gradual decrease in the γG globulin content as well as in the titre to gonococcal antigen The reduction in titre was estimated to correspond fairly well to the decrease in γG globulin concentration After two absorptions with a reactive staphylococcal strain complement fixing gonococcal antibodies were still detectable whereas γG globulin was not detectable by immunoelectrophoresis This might be explained by a greater sensitivity of the complement fixation test and/or to antibody activity of remaining γM globulins

The protein nature of the receptor on the surface of the reactive staphylococci was indicated by its sensitivity to trypsin[1] *Forsgren & Sjoquist* (5) investigated the reaction of the staphylococcal antigen Protein A with human γG globulin and found a degree of affinity similar to that found in the present study between reactive strains and rabbit γG globulins Furthermore the affinity between γG globulins and the reactive staphylococcal strains did not seem to be limited to rabbit globulins In the F A T a similar reaction was found between those strains and FITC labelled globulins from guinea pigs mice and sheep (13) Thus the reactive strains might be strains with a large content of Protein A *S aureus* Cowan type I which was known to possess large quantities of Protein A (8 15) was therefore included in the first absorption experiments with normal human serum The results supported our concept since 1) *S aureus* Cowan type I absorbed γG globulin just as selectively as did the known reactive strains and 2) absorption of human serum gave quantitative and qualitative results similar to those found after absorption of rabbit sera

The experiments of *Forsgren & Sjoquist* (5) indicate that the binding of Protein A to human γG globulin involves another part of the γG globulin molecule than the active site within the Fab fragment of acquired antibodies It is possible that the site is located in parts of the Fc fragment which are common to human γG globulin molecules of different antibody specificity and perhaps also common to γG globulin molecules of other species This interpretation may explain many of the unexpected and peculiar findings in the serology of staphylococci (*e g* 3 4)

Kronvall (10) used a living strain of *S aureus* Cowan type I as source of Protein A for absorption experiments with a γG myeloma globulin before and after treatment with papain The myeloma globulin possessed antistreptolysin activity (AST activity) When the γG

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[1] Unpublished experiments performed in collaboration with *Kirsten Rosendal* M D Department of Diagnostic Bacteriology Statens Seruminstitut

myeloma globulin solution was absorbed the AST activity was removed. However, after absorption of the papain digest by which only the Fc fragment was removed, the AST activity remained in the solution. Thus, Protein A or reactive strains might be used as a means of separating the fragments of γG globulin molecules.

It was shown recently (6) that γG globulin aggregated with Protein A may have a biological significance similar to that of antigen-antibody complexes.

The second set of experiments with normal human serum served to elucidate the effect of formalin treatment of the bacteria. Absorptions were performed with three batches of a reactive strain which were treated with different concentrations of formalin for varying periods of time. Only the strongest formalin concentration (5 per cent for 24 hours) changed the outcome of the absorption by including absorption of γA and γM globulins. The amount of absorbed γG globulin was reduced simultaneously. The absorption effect of such an antigen is presumably comparable to that of different tissue powders used for absorption of conjugates for the F.A.T. These findings are not incompatible with those of *Prochazka* (18) who showed that formalin treatment is more damaging to the non-specific reaction of staphylococci than to a specific antigen-antibody reaction.

SUMMARY

Normal rabbit serum, homologous and heterologous rabbit antisera and normal human serum were absorbed with reactive and non-reactive staphylococcal strains. Analysis of the sera by immunoelectrophoresis, agar gel electrophoresis and double diffusion in agar a.m. Ouchterlony before and after absorption showed that the affinity of the reactive strains was predominantly limited to γG globulins. Non-reactive strains caused minor and presumably insignificant changes.

Possible identity between the phenomena described for reactive strains and the reaction between Protein A from *S. aureus* and human γG globulin described by *Forsgren & Sjoquist* (5) is discussed.

ADDENDUM

After the completion of this investigation the Swedish group of workers has published results of further studies on "Protein A" (*Forsgren, A. & Sjoquist, J.*: Protein A from *Staphylococcus aureus*. III. Reaction with Rabbit γG globulin. *J. Immunol.* 99, 19-24, 1967). The results reported confirm that the reaction between "Protein A" and rabbit γG globulin is analogous to that found between "Protein A" and human γG globulin, and that the part of the γG globulin molecule involved presumably is located in the Fc fragment.

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DEMONSTRATION OF *YERSINIA ENTEROCOLITICA* BY THE FLUORESCENT ANTIBODY TECHNIQUE

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Received 2 1 68

Recent investigations of *Yersinia enterocolitica* and human intestinal disease with symptoms suggesting appendicitis has stimulated interest in this organism (*Hassig et al* 1949 *Carlsson et al* 1964 *Mollaret & Chevalier* 1964 *Wauters & Mollaret* 1965 *Winblad & Sternby* 1966 *Graux & Wauters* 1966 *Winblad Nilehn & Jonsson* 1966 *Winblad, Nilehn & Sternby* 1966 *Niléhn & Sjostrom* 1967) In many serologically positive cases the organism has been isolated from the intestinal flora A reliable culture technique has been elaborated (*Winblad Nilehn & Jonsson* 1966) But like species determination based on the biochemical properties of the bacterium it is fairly time consuming

In an attempt to devise a useful fluorescence immunological technique for demonstrating *Yersinia enterocolitica* in human material especially the faeces a fluorescein isothiocyanate labelled anti Yersinia enterocolitica globulin was studied for its specificity (*Cederberg* 1967a 1967b) The unabsorbed conjugate together with lissamine rhodamine conjugated antistaphylococcal serum proved highly specific It was thought that it could be used in routine laboratory work

The method was therefore now tried out on a number of faecal samples and the results were compared with those obtained by conventional culture

MATERIAL AND METHODS

Faecal samples The material consisted of 678 samples collected within a 4 month period from 518 patients with various abdominal symptoms especially diarrhoea and symptoms simulating those of appendicitis The patients belonged in the material of a concurrent investigation of *Yersinia enterocolitica* in abdominal diseases (*Niléhn & Sjöström* 1967)

Culture Cultures were made on all samples The technique described by *Winblad Nilehn & Jonsson* (1966) and *Nilehn & Sjöström* (To be published) was used[1]

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[1] Cultures and biochemical identifications of all strains isolated were made by Dr *B* Nilehn

All Y *enterocolitica* strains isolated were typed by agglutination technique using absorbed immune sera from rabbits immunized with O antigen (*Winblad* 1967). They were also tested by FA technique using strain Winblad conjugate (*Cederberg* 1967b).

Preparation of conjugate Rabbit anti sera were prepared by immunization with live *Yersinia enterocolitica* human type strain Winblad (*Carlsson et al* 1964). The anti globulin was conjugated with fluorescein isothiocyanate (FITC). Anti staphylococcal serum was prepared by immunization of rabbits with *Staph aureus* (strain 193 phage type 52/80) and conjugated with lissamine rhodamine B (RB 200). The immunization and conjugation procedures have been described in a previous publication (*Cederberg* 1967b).

The homologous bacterial strain was used for titrating the conjugate. The highest dilution giving maximum fluorescence intensity (4+) was used as a working dilution. It was usually 1 80–1 160.

Preparation and staining of faecal smears A small amount of faeces was taken with a cotton swab and suspended in physiological saline smears were made on slides which were dried in the air and heat fixed. The preparations were used the same day as the samples were received at the laboratory. Some smears were kept together with the suspensions at —20 C for control and supplementary examination if desired. The smears were stained by adding one or two drops of a mixture containing equal parts of anti Yersinia conjugate diluted according to the previous titration and undiluted anti staphylococcal conjugate (RB 200 labelled) as a counterstain. After incubation for 30 minutes at room temperature the slides were washed in phosphate buffer pH 7 8 and mounted with a cover slip and glycerine buffer.

Two hundred consecutive specimens included in the material were investigated also after incubation in broth. A small amount of the specimen was inoculated into Rappaport broth (*Rappaport et al* 1956 1959) which was incubated at 37 C for 18–20 hours. Smears were then made and fixed and stained in the way described above (Delayed FA test).

Fluorescence microscopy The equipment used was described in a previous paper (*Cederberg* 1967b).

Fluorescence intensity was graded from 4+ (strong fluorescence with brilliant margins of the bacterial cell) to 1+ (weak diffuse fluorescence) and 0.

Controls One smear from each sample was stained with anti Yersinia conjugate and RB 200 conjugate as described above. If the result was positive the procedure was repeated with another smear and two control smears tested by staining with

1) FITC conjugated globulin from a non immunized rabbit (+ RB 200 conjugate)
2) Anti Yersinia conjugate in double working concentration mixed with an equal volume of undiluted unconjugated anti Yersinia serum (+RB 200 conjugate) (One step inhibition test)

The FA test was judged as positive if fluorescent bacteria (intensity at least 3-4+) were found with anti Yersinia conjugate no fluorescent bacteria were found with control conjugate and no fluorescent or very weakly fluorescent bacteria were found in the inhibition test.

Serology Most of the bacteriologically positive cases were investigated by repeated estimation of the agglutinin titre against Y *enterocolitica* (human type O antigen). The technique described by *Winblad Wehn & Sternby* (1966) was used. A case was said to be serologically positive if the titre rose by at least two steps in course of the illness.

RESULTS

Among the 678 faecal specimens (from 518 cases) examined with the FA test and culture 22 (from 17 cases) proved positive by both methods and 645 negative. Six specimens were positive as judged by the FA test but negative on culture. Five specimens were positive on culture only (Table 1). These 11 specimens derived from 9 cases. Table 2 shows the results of FA tests and cultures and serology in these cases.

In all but two of these cases the bacteriological diagnosis was confirmed by culture from one or more specimens From all but two of the cases one or more FA positive specimens were obtained Examination of a second smear from one of the specimens primarily judged as FA negative resulted in a positive FA test Only a small number of fluorescent bacteria were observed in the specimens obtained from the two cases where cultures were negative One of these cases was serologically negative The other was not studied serologically

TABLE 1

Comparison of Results Obtained with FA Technique and Culture Used with a View to Detecting Y enterocolitica in 678 Faecal Samples

Results of FA test and culture	Number of samples
FA test + Culture +	22
FA test + Culture —	6
FA test Culture	5
FA test — Culture —	645

TABLE 2

Investigation of 9 Cases Including those in which Results of FA Test and Culture were Discordant

Case	Sample No 1 FA	Sample No 1 Culture	Sample No 2 FA	Sample No 2 Culture	Sample No 3 FA	Sample No 3 Culture	Sample No 4 FA	Sample No 4 Culture	Serology
M K	+	—	+	+					Positive
M H	+	—	+	+	—	—	—	—	Positive
M E	+	+	+	—	—	—			Positive
S S	+	—							Not done
B D	+	—		—					Negative
T S		+	—	—	+	—	—	+	Positive
E A	+	+	+	+	—	+			Positive
S J	—	+							Not done
M C	—	+							Positive

A second smear from the same sample FA+

All of the 27 Y *enterocolitica* strains isolated reacted strongly with the conjugate used in this investigation They belonged to the same O antigen type (Winblad 1967) as shown by agglutination technique

The 645 negative specimens included 34 with very small but usually numerous fluorescent bacteria showing intense solid staining and not the marginal staining typical of Ye a Because of the smallness of their size less than half that of *Yer* their coccoid appearance and

their solid staining they were readily distinguished from Yersinia. They did not stain with the control conjugate (from non immunized rabbit). In the one step inhibition test they behaved like *Yersinia enterocolitica*. Judging from their appearance in the dark field and fluorescence illumination these strains were identical. It proved possible though with difficulty to isolate 3 of these strains. All were fastidious gram negative anaerobic cocci growing in colonies barely detectable with the naked eye. They could not be identified with certainty but in some respects they resembled Veillonella. Their immunological resemblance to *Y. enterocolitica* has been discussed in detail in a previous paper (*Cederberg* 1967 b).

The 34 specimens containing Veillonella-like organisms derived from 32 cases. Eighteen of these cases were studied serologically with determination of the agglutinin titre against *Yersinia enterocolitica*. Fifteen had no demonstrable titre. In 2 the titre increased significantly but in one of them *Yersinia enterocolitica* had been demonstrated in a sample obtained a few days earlier. A third patient had six months previously had a high Yersinia agglutinin titre and *Yersinia enterocolitica* but no Veillonella-like organisms had repeatedly been demonstrated in the faeces. Six months later when the Veillonella-like were seen no increase was noted in the Yersinia agglutinin titre which was then only 1:20.

Two hundred of the faecal samples were examined both with the direct and the delayed FA test. The latter was performed the following day with a smear made from Rappaport broth inoculated with a small amount of the sample. Ten specimens proved positive by both methods. One was positive as judged by the direct test but negative by the delayed test. None was positive by the delayed test alone and only one specimen yielded a substantially larger number of fluorescent bacteria in the delayed than in the direct test.

DISCUSSION

In a recent investigation (*Cederberg* 1967 b) it was found that all of 12 strains of *Yersinia enterocolitica* isolated from human material reacted strongly with conjugates obtained from rabbits immunized with a live human strain (strain "Winblad").

Since then a further 30 human strains have been tested. All stained uniformly with the same conjugate (*Cederberg* 1967 a, *Cederberg* unpublished data). This was therefore regarded as satisfactory for the present investigation. Later 3 strains have been isolated which do not stain with this conjugate. Their species identity is debatable. According to *Winblad* (1967) these strains constitute two O antigen types separate from the other 75 human strains, strain "Winblad" included, all of which belong to the same O antigen type.

If the immunofluorescent technique is to be used for the demon

stration of bacteria it must have certain practical advantages over the conventional culture technique Moreover the method should be at least just as reliable Isolation and biochemical identification of *Yersinia enterocolitica* from the faecal flora requires at least 4 days The FA method is simpler and requires only a couple of hours In some cases the results of the two methods were discordant (Table 2) When the FA test was positive but the culture negative this may be explained by the greater sensitivity of the FA method and its ability to identify small numbers even of dead organisms The possibility of a false positive diagnosis by the FA test is less probable because several patients earlier or later yielded culture positive samples and were serologically positive

Negative FA tests from samples giving positive culture cannot be explained by incapacity of the bacteria to react with the conjugate because the contrary was afterwards proved with the isolated organism It may be due to uneven distribution of the organisms in the specimen In one case there was a negative FA test as judged from the first smear but positive from a second smear from the same sample Only a small number of fluorescent bacteria were observed in this smear Uneven distribution of the bacteria may also explain why a negative culture can be seen when the FA test is positive

If the FA test is used as a quick and labloursaving method time consuming enrichment procedure is hardly desirable Preliminary experiments in which the sample was incubated 1 day in Rappaport broth did not suggest that this would imply any advantage A growth medium with higher selectivity is as yet not known The counterstaining technique used proved to facilitate the evaluation of the preparation It not only blocks the staphylococci and several other bacteria against which antibodies normally occur in the rabbit serum but also stains them as well as the leucocytes and other non bacterial components of the intestinal content which take on a red brown colour contrasting with the apple green FITC fluorescence The technique has been used successfully by *Danielsson* (1965) for demonstration of gonococci

Of greatest interest in the serology of *Yersinia enterocolitica* is of course the question whether the above mentioned Veillonella like organisms give rise to any antibody reaction From an immunological point of view they closely resemble *Yersinia enterocolitica* However the investigation produced no evidence suggesting that they produce antibody reactions

It would obviously be desirable to use a conjugate where this cross reaction with the Veillonella like organism had been eliminated Because of the extremely scanty growth of this organism on artificial media it has not been possible to use it for absorption of the Yersinia conjugate However as was shown in a previous investigation (*Cederberg* 1967b) it was possible to inhibit the homologous Yersinia fluorescence completely with the aid of anti "Veillonella serum It would thus

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BRIEF REPORT

AGE DISTRIBUTION OF ANTIBODIES TO BURKITT CELLS

By *Arne Svedmyr* and *Aberra Demissie*

Antibodies to a herpes like virus referred to as EB virus and originally found in cell lines derived from Burkitt lymphomas have been reported to be widely distributed (*Henle & Henle* 1966) and to develop regularly in connection with infectious mononucleosis in American patients (*Niederman et al* 1968) A survey of the age distribution of antibodies to a Burkitt cell line in a Swedish population is reported here

Materials and Methods

The *serum specimens* investigated were collected in 1965 for a study of the rubella immunity among females in the town of Eskilstuna (*Lundström et al* 1967) The specimens were inactivated at 56 C for 30 min and stored at —30 C.

Cells The Silfere line derived from a Burkitt lymphoma was kindly supplied by Dr George Klein It was employed throughout the study reported here The cells used were free from mycoplasma Eagle s basal medium containing double amounts of arginine 20 per cent inactivated (56 C 30 min) postnatal calf serum and antibiotics was used The cells were grown in a 1 litre flat Roux flask containing 150 ml of medium The flask was rubber stoppered and incubated without gassing in a horizontal position at 35 C It was gently shaken about once daily The medium was changed twice weekly At the same time about one third of the cell sediment was transferred to a 200 ml bottle with 25 ml of the same medium but prepared without arginine (*Niederman et al* 1968) This flask was kept at 35 C in a 5 per cent CO atmosphere for 9 days The cells were then washed twice with PBS placed on microscope slides air dried and fixed with acetone at room temperature for 10 minutes The slides were stored at —30 C until used

Staining The cell smears were first exposed to serum diluted 1/8 or more for one hour at room temperature After thorough washing they were overlaid with an appropriate dilution of a fluorescein conjugated sheep antihuman gammaglobulin and again incubated and washed as above After mounting in 90 per cent buffered glycerine the preparations were examined under ultraviolet illumination (Osram HBO 200 W lamp) with a Leitz microscope

At every staining a control was made with a 1/100 dilution of a Burkitt serum with a titre of 1/2560 It was kindly provided by Dr George Klein

TABLE 1

Antibodies to Burkitt Cells and to Rubellavirus as Correlated to Age

Age (years)	Newborn	2	5	10	15	20	25	30	≥ 40
Nr investigated	35	34	35	35	33	33	35	32	35
Nr Burkitt reactive	32	4	15	24	22	31	30	30	31
% Burkitt reactive	91	12	43	69	67	94	86	94	83
% with rubella NT antibody (35 investigated per group)	83	0	14	51	57	74	89	94	97

From Lundstrom et al. (1967)

Results

As seen from Table 1 antibodies to intacellular antigen(s) of the Burkitt cell line used are acquired in a similar pattern as the immunity to common viral dis

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cases of childhood although somewhat earlier than the immunity against rubella virus. The results agree well with those just published by *Henle & Henle* (1967). Whereas the control serum from a Burkitt patient used at 1/100 dilution stained between 9.1 and 10.9 per cent of the cells, both the frequency and the brilliance of stained cells were usually less with the positive Swedish sera although diluted 1/8, the frequency ranging between 0.4 and 11.0 per cent. End point titres of 36 sera representing different degrees of staining varied between 1/8 and 1/1024. The frequency of stained cells as well as the brilliance decreased gradually with serum dilution.

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THE ISOLATION OF AN OXIDASE-NEGATIVE STRAIN OF *PSEUDOMONAS AERUGINOSA* FROM THE URINE OF A COBALT-IRRADIATED PATIENT

By *J. Holmgren*

In 1956 *Kovács* described a sensitive oxidase reaction for the identification of Pseudomonas. He examined 436 strains of *Pseudomonas aeruginosa*, all of which showed a positive oxidase reaction. These results were confirmed by *Buttiaux & Gagnon* (1958) who also pointed out that the Shewan antibiotic test is useful for a differentiation between *Pseudomonas* and *Achromobacter*. *Pseudomonas* is resistant to penicillin while *Achromobacter* is sensitive.

The constant oxidase positivity of *Pseudomonas aeruginosa* has made the oxidase test a routine test in most diagnostic laboratories. More than 2000 strains of *Pseudomonas aeruginosa* isolated from samples sent to our laboratory have been tested for the presence of oxidase with Kovács's method. All the strains have given a positive oxidase reaction. However, recently we isolated one strain which was oxidase negative.

Materials and Methods

The strain in question, labelled Ps ST, was isolated from a sample of midstream urine. Tests for the presence of oxidase were performed as described by *Kovács* (1956) with the use of 1 per cent tetramethyl-p-phenylenediamine aq. solution as reagent. Tests were also performed with the more stable dimethyl-p-phenylene-diamine as described by *Leclerc & Beerens* (196).

Results

The strain Ps ST was found to consist of Gram-negative motile rods. The appearance on culture media was typical for *Pseudomonas aeruginosa* with production of bluish green pyocyanin pigment diffusing into the medium. The characteristic sweetish aromatic odour could also be observed. Ps ST was resistant to penicillin.

Oxidase tests were negative both with dimethyl- and tetramethyl-p-phenylene-diamine reagent.

The properties of Ps ST are summarized in Table I.

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TABLE 1
Characteristics of the Oxidase Negative Strain Py ST

Motility	+	Sucrose ferment	—	H_2S formation	—
Pyocyanin	+	Lactose ferment	—	Catalase	+
Odour	+	Mannitol ferment	—	Urease	+
Glucose ferment	+	Indol formation	—	Oxidase	—

The medical record of the patient from which the oxidase negative strain Py ST was isolated offers interesting information. The patient is a male 67 years old who in April 1967 suffered from haematuria. His urine then contained oxidase positive *Pseudomonas aeruginosa*. During the summer 1967 a left side hypernephroma was diagnosticated and removed. After the operation the patient was treated with cobalt irradiation. This treatment consisted of five irradiations (400 r each directed towards the operation area at a distance of 60 cm) with intervals of two days between the irradiations. The treatment was finished on the day the urine sample containing Py ST was collected (July 18th 1967).

It is a well known fact that X- and other radiation can induce mutations in bacteria. It is tempting therefore to suspect the cobalt irradiation of the patient to be responsible for the demonstrated lack of oxidase.

Summary

The observation of an oxidase negative strain of *Pseudomonas aeruginosa* isolated from the urine of a cobalt irradiated patient is reported. It is discussed whether this cobalt irradiation treatment may have induced the lack of oxidase.

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ESTERASE ACTIVITY OF ANTICOMPLEMENTARY MEDIA OF NONINFECTED HeLa CELL CULTURES

By *Gerolf von Zeipel*

In a previous work (*von Zeipel* 1958) *noninfected* HeLa cells were found to render serumfree maintenance media anticomplementary, the active principle being non-dialyzable and heatlabile. Addition to the media of heat inactivated serum from some animal species prevented the development of anticomplementary activity or abolished it if it already formed. This heatstable serum factor seemed to move electrophoretically among the α globulins.

In the present paper some evidence of the enzymatic nature of the anticomplementary activity will be presented.

Materials and Methods

HeLa cell cultures were prepared as described earlier (*von Zeipel* 1958). Maintenance media (Parker 199 + additions) of Roux bottle cultures were harvested after 6 days of incubation and centrifuged at low speed followed by 60 min in a

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Spinco type 30 rotor at 30 000 rpm. The supernatant was concentrated 75 fold on a Diaflo XM 50 membrane (Amicon, U.S.A.) and finally run for 60 min at 40 000 rpm in a Spinco type 50 Ti rotor. The supernatant was stored at —30 C.

Anticomplementary activity was estimated as described previously (*von Zeipel* 1958). Titres are given as the reciprocal of the dilution contained in 0.02 ml which still inhibited two full units of complement.

Esterase activity was tested according to Ratnoff and Lepow 1957 on N acetyl-L-tyrosine ethyl ester (ATEe, Brit. Drughouse, England) and on p-toluene sulphonyl-L-arginine methyl ester (TAMe, Calbiochem, Switzerland). Hydrolysis of the esters was followed at 37 C with an autoburette of a pH stat titration apparatus (Radiometer, Denmark). Acid liberated was neutralized with 0.05 N NaOH. The total reaction volume of 2.5 ml contained 0.02 M ester and anticomplementary material in a 0.005 M phosphate buffer with 0.15 M NaCl (pH 7.5). Esterase activity is expressed in micromoles of acid produced per ml of preparation and hour.

Inhibitors. Soybean (SBTI, 2 × crystallized) and ovomucoid trypsin inhibitors (Sigma, U.S.A.), Trasylol (Bayer, Germany), Diisopropylfluorophosphate (DFP) was a gift from the Research Institute of National Defence, Sweden. The inhibitors *mentioned were incubated with anticomplementary samples for 2 hours at 37 C* followed by 18 hours at +4 C. Guinea pig and human sera were stored at —65 C until used.

Gel filtration tests were performed on Sephadex G 200 (Pharmacia, Sweden). *The elution buffer (pH 8 or 9) contained 0.05 M trishydroxymethylaminomethane* 0.1 M NaCl and 0.02 per cent NaN_3. Fractions dialysed against the above phosphate buffer were tested for esterase and anticomplementary activities.

Results

The ATEe activity of concentrated media usually was about 50, the anticomplementary titres being 300 to 600. The activity, having an optimum about pH 7.5, was *high between pH 6 and 8.2. Beyond these limits a pronounced decrease was* noticed.

The hydrolysis of TAMe was 85 to 90 per cent lower than that for ATEe.

Anticomplementary activity was more impaired by heat treatment than was esterase activity. *During incubation for 30 min* 100 per cent of the anticomplementary activity was inactivated at 56 C, 96 per cent at 50 C and 50 per cent at 45 C. The corresponding reduction of esterase activity was 93 per cent at 60 C, 76 per cent at 56 C, 73 per cent at 50 C and 25 per cent at 45 C.

The esterase and anticomplementary activities of samples containing 1.7 mg of protein remained unchanged upon treatment with 1000 units of Trasylol or 2 mg of either SBTI or ovomucoid inhibitors.

Anticomplementary activity was inactivated by DFP at a concentration of —2.5 M to 100 per cent, at —3 M to 98 per cent, at —3.5 M to 94 per cent and slightly at —4 M. The corresponding reduction of esterase activity was —2.5 M 86 per cent, —3 M 80 per cent, —3.5 M 73 per cent and —4 M 35 per cent.

Fresh human serum inhibited esterase activity immediately whereas inactivated serum had only a moderate effect even after preincubation at 37 C for one hour. Inactivated guinea pig serum on the other hand showed a strong inhibition which increased on incubation. Fresh guinea pig serum behaved similarly as the inactivated one (56 C, 30 min).

On Sephadex filtration esterase and anticomplementary activities were eluted in the same fractions and showed a migration rate similar to that of alkaline phosphatase of calf intestinal type which has been attributed a molecular weight of 115 000 (*Andrews* 1964).

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